THE ESTERS OF CHOLESTEROL

Constitution of Their Fatty Acid Component in Human Serum.

by

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CONTENTS

Preface

This work was undertaken with the object of identifying the fatty acid component of the cholesterol esters present in human blood serum.

Ignorance of the precise nature of these acids hampers the understanding of the role of cholesterol in the metabolism of the human organism and is also a stumbling block to the development of a sound method for the estimation of blood cholesterol levels.

In the experiments described here, three samples of serum and one of plasma have been examined; two of the sera were obtained by combining specimens from several patients in the acute wards of a general hospital, the third came from a healthy adult male aged thirty-nine years; the specimen of plasma was obtained from transfusion bank blood and may be presumed to have come from healthy donors.

The lipids were extracted from the blood specimens by conventional methods and the cholesterol esters were separated from the other lipids by adsorption chromatography on columns of silicic acid. After hydrolysis of the esters, the fatty acids recovered were examined by reversed phase chromatography on columns of medicinal paraffin. With this technique, increasing strengths of aqueous acetone are used to elute different fatty acids; the longer the chain length, the greater is the strength of acetone required for elution.

Groups of acids separated by this process were then subjected to catalytic hydrogenation and again chromatographed in order to determine their respective chain lengths. Comparison of their chromatographic behaviour before and after hydrogenation indicated their degree of unsaturation, since unsaturated acids are eluted with lower strengths of acetone then saturated acids of the same chain length. Finally, portions of the acids recovered in two of the experiments were exposed to oxidation by alkaline potassium permanganate, and the recovered products examined chromatographically. The oxidation products of the unsaturated acids are immediately eluted whilst the unchanged saturated acids are eluted in their usual positions.

The results obtained from these experiments showed that the principal components were oleic and linoleic acids which were present in approximately equal amounts and together accounted for about 60% of the total. About 27% was found to consist of saturated acids, whilst the remainder consisted of small amounts of highly unsaturated acids.

I am indebted to Mr. R.F. Nunn, B.Sc., F.R.I.C. of the Royal Sussex County Hospital, Brighton and St. Francis Hospital, Haywards Heath for advice, encouragement and helpful criticism, and also for providing most of the purified acids used in the preliminary work. I also wish to thank Mr. D. Budgeon of St. Francis Hospital for constructing the thermostatic jacket for the reversed phase column and the apparatus for alkaline isomerisation. Thanks are also due to Messrs. Unilever Ltd. of Port

Sunlight for a gift of purified linoleic acid.

INTRODUCTION

INTRODUCTION

Cholesterol, the parent compound of all the animal sterols, was discovered almost simultaneously by Pouletier de la Salle about 1770 and Conradi in 1775. (Elsevier 1940, Sobotka 1938) It is of widespread distribution in the body but after almost two centuries, knowledge of its purpose there is small.

It is a complex unsaturated secondary alcohol and like other alcohols it can form esters both with organic and inorganic acids, but naturally occurring esters appear to be formed only by its combination with fatty acids. Natural esters were first isolated from serum by Hürthle (1896) and a method of quantitatively separating natural mixtures of the alcohol and ester by precipitation of the former with digitonin was devised by Windaus (1909). With the aid of this method the distribution of the two forms in the body was soon charted. The free alcohol was found to be present in significant amounts in almost every tissue in the body with brain predominating, cholesterol being responsible for more than one tenth of the dry weight of this organ. (Dorée, 1937)

The ester is much more limited in its distribution, being confined to the liver, the cells of the intestinal mucosa, the adrenal and reproductive glands, and the blood serum and chyle. The ratio between the free alcohol and the ester in blood serum is remarkably constant; the free being about one third of the total. This ratio is

unaffected by diet or by changes in the serum cholesterol level, though in a small number of diseases the ratio may be changed; for example, in hepatitis and secondary xanthomatosis, the relative amount of free cholesterol may be increased. (Peters and Van Slyke, 1946 b & c)

In spite of numerous investigations in the field of cholesterol metabolism, the nature of the fatty acids esterified with cholesterol in human serum has never been established though evidence has accumulated to show that they are highly unsaturated. The barriers to further information have always been the difficulty of isolating cholesterol esters free from other acid bearing lipids, and the lesser but still great difficulty of identifying the component acids in the small quantities which are usually available.

There is great need to complete our knowledge of these compounds for in this knowledge lies an essential clue to some at least of the functions of cholesterol. Great advances have been made in the understanding of the cycle of synthesis and degradation of the sterol itself, beginning with the demonstration by Bloch in 1942 that deuterated acetic acid was incorporated in the cholesterol molecule in vivo. Since then, most of the steps in the synthesis both of the nucleus and the side chain have been exposed.

Progress towards the explanation of the purpose of cholesterol has not kept pace with these advances although

reasonable theories have been put forward. Bills (1935) suggested that the more important activities of the sterol included fat transportation, insulation of nerve sheaths, effects on cellular permeability and neutralisation of toxins. A relationship between cholesterol and the bile acids has been shown and it is now accepted that cholic acid is formed from cholesterol (Bloch et al, 1943). The biological conversion of cholesterol to pregnandiol has also recently been proven (Bloch, 1945).

Recent clinical and pathological studies, most notably those of Bronte-Stewart and his colleagues (Bronte-Stewart et al, 1955) have shown a relationship between dietary fat, blood cholesterol levels and disease of the coronary arteries. The effect of the so-called "essential" fatty acids in lowering the blood cholesterol, when included in the diet for long periods, has been recorded by numerous observers (Gordon et al, 1957, Malmros et al, 1957). The compounds concerned, linoleic and arachidonic acids, possess two and four double bonds respectively and are essential in the diet inasmuch as only one double bond can be created (or destroyed) by the body itself (Schoenheimer and Rittenberg, 1936 a & b). The function of these acids in the human is obscure since it is, apparently, impossible to produce a deficiency in man. Even in extreme starvation the essential acids are jealously preserved (Sinclair, 1935). This careful conservation of these acids strongly suggests that they are of great importance, whilst animal experiments have shown that diets

deficient in them result in skin disorders, failure to grow and renal damage.

Unsaturated fatty acids are extremely chemically reactive, readily forming hydroxy compounds or breaking at the double bond to form shorter chains (Finar, 1951, Ellis, 1950). This process, occurring naturally, forms part of the phenomenon of rancidity (Bloor, 1943a). These chemically active substances are not stabilised when constituted as triglycerides or soaps and could be expected, both, to exert a toxic action in the body, and to be rapidly destroyed. It follows that some means of maintaining them in a stabilised state must exist. With these factors in mind, the high degree of unsaturation of the cholesterol esters seems to be more than coincidence.

The problem is of some importance also to the clinical laboratory, where the methods in use for the estimation of serum cholesterol are legion and the ranges of results which they give equally numerous. It is seldom possible to make accurate measurements of an unknown substance and reliable techniques can only be developed when a structure is fully known.

The first work in this field appears to have been that of Bloor (1924) who obtained partial separation of the plasma lipids by precipitating the phospholipids with acetone and then subjecting the residual mixture of triglycerides and cholesterol to fractional crystallisation from ethanol. Despite the crudity of these experiments

this author was able to show that the acids of cholesterol were the most unsaturated, those of the triglycerides were the least unsaturated, whilst those of the phospholipids were of intermediate degree. This finding has survived to the present day unchallenged save by Cahn and Houget (1933, 1935) whose theories were not supported by any convincing experimental data.

No advances were made until 1941 when Kelsey and Longnecker were able to identify the fatty acids of beef plasma lipids. These workers employed a system of selective enzymic hydrolysis developed by the former (Kelsey, 1939, 1,11,111). The phospholipids were precipitated with acetone as in Bloor's experiment and the residues were treated with a modified intestinal lipase to split the triglycerides. Unfortunately acetone does not precipitate all the phospholipids (Lovern, 1955a) and moreover the lipase preparation used gave incomplete hydrolysis of triglycerides when used on serum extracts, although it was effective when used with artificial mixtures. The method also suffers from the disadvantage of requiring large amounts of material; indeed the authors employed no less than forty two litres of plasma in their original experiment. Despite these defects the marked unsaturation of the cholesterol acids noted by Bloor was confirmed, and the presence of hexadecenoic, oleic, linoleic, linolenic, and arachidonic acids demonstrated.

Saturated acids in the form of palmitic and stearic acids were also found to be present.

The difficulty in separating substances as similar in properties as the triglycerides and cholesterol esters demands a highly selective method which has been made available by recent advances in chromatographic techniques. In 1940 Trappe used columns of alumina to separate cholesterol esters from the free alcohol for the quantitative measurement of these two fractions. Hess (1947) extended this work and it was further developed and confirmed by Kerr and Bauld (1953). In 1952 Borgström was able to separate phospholipids and cholesterol esters from a mixture of them with stearic acid and triglyceride by passing them down columns of prepared silicic acid. Clément et al (1954), developing the method devised by Trappe, used highly activated alumina to separate cholesterol esters in an attempt to support the theories of Cahn and Houget already mentioned. They found that the separated cholesterol ester fatty acids possessed a high iodine value (at least 130) and concluded that Bloor's original findings in 1924 were correct. A more elegant method of separating plasma lipids on silica columns was developed by Fillerup and Mead (1953, 1954). Using this technique all major groups of fats in serum extracts can be separated without any additional treatment.

Chromatographic methods have also been developed which permit the separation, identification and approximate measurement of the individual members of a mixture of fatty acids.

Notable work in this field includes the separation of C2-C8 acids on columns of buffered silica gel by Moyle and her associates (1948), separation of C6-C22 acids on reversed phase columns of rubber (van de Kamer et al 1955), and, probably the most important, reversed phase partition chromatography of C12-C18 acids on columns of medicinal paraffin by Howard and Martin (1950). The Howard and Martin method was elaborated and extended to include acids of chain length up to C24 by Silk and Hahn (1954).

The experiments described hereafter employ certain of these chromatographic methods to identify the fatty acids of the cholesterol esters in human serum.

EXPERIMENTAL DETAILS

1. Outline of Experiments.

2. Analytical Procedures.

1. Outline of Experiments

The methods used are given here in general terms; full details of the analytical procedures are recorded in the subsequent section.

The major procedures involved in this work fall into four parts.

(1) Extraction of lipids from serum.

(2) Chromatographic separation of the cholesterol esters from the other lipids on silicic acid columns.

(3) Hydrolysis of esters and subsequent extraction of fatty acids.

(4) Chromatographic separation and identification of fatty acids on reversed phase partition columns of medicinal paraffin.

In the four experiments, although they were basically similar, the emphasis was placed on different aspects.

Experiment 1. was a pilot experiment and great care was taken to avoid degradation of the fatty acids even at the expense of poor recoveries.

In Experiment 11. the recovery of material from the silicic acid columns was fully investigated and the constitution of the recovered fats examined in greater detail.

Experiment 111. was arranged to provide a large amount of material so that the nature of the recovered acids could be more fully determined.

Experiment IV was undertaken to show that fresh serum gave results comparable with those obtained from the other specimens.

The conclusions are based upon the results obtained in Experiments 11, 111 and IV.

Three samples of serum and one of plasma were used. Sample 1 was of 80 ml. volume and was obtained by combining small samplesof serum from twenty-five patients in the acute wards of a general hospital, patients with known liver disease being excluded. Sample 11 was of similar origin from 25 patients but was of 100 ml. volume. None of the constituent portions of samples 1 and 11 was more than 48 hours old when extraction was commenced, and all constituent portions were stored in the refrigerator at 0°C. until used. Sample 111 consisted of the plasma from two pints of transfusion bank blood withdrawn during the process of preparing packed cells. This was stored at 0°C. for six weeks before extraction. This sample was of course diluted with disodium hydrogen citrate and dextrose sdution, which provided about one quarter to one third of its volume. Sample IV was a single specimen of serum of 25 ml. volume from a healthy male aged 39 years. The subject was not fasting when the sample was withdrawn and the serum was separated and extraction commenced within two hours of withdrawal.

(1) The Extraction of Lipids

The extraction procedures varied to some extent with the samples. Sample 1 was extracted at temperatures of 37°C. or lower. The initial process was carried out at

room temperature, the serum being poured slowly, with shaking, into 250 ml. of ethanol and the protein residues, after filtration, re-extracted twice with ethanol ether 1:1 first with 150 ml. and then with 100 ml. The combined extracts were evaporated to small volume on a water bath at 37°C. and the residual liquor extracted three times with acetone 100 ml. saturated with magnesium chloride. The pooled acetone extract was filtered and evaporated almost to dryness again under reduced pressure at 37°C. and the residue extracted three times with petroleum ether (B.P. 40°-60°) 150, 100 and 50 ml. This extract was dried overnight over anhydrous sodium sulphate and then evaporated to dryness.

Samples II and IV were extracted in a similar fashion to each other. The serum was poured into ten times its own volume of ethanol, allowed to stand overnight and filtered. The protein residues were dispersed in chloroform methanol 1:1 in amount equal to the original volume of serum, refluxed for three hours and filtered. This was repeated and after the final filtration the residues were washed twice with one volume of chloroform. The pooled extracts were evaporated to small volume at reduced pressure at temperatures below 60°C. on the water bath. The residues were extracted three times with petroleum ether (B.P.40°-60°) in amount equal to the original volume of serum. The combined extracts were dried over sodium sulphate.

Sample III was poured into twice its volume of ethanol and allowed to stand overnight, the protein residues from

filtration being then refluxed with ethanol ether 1:1 in amount equal to the original volume of plasma. After again filtering, the protein residues were dispersed in chloroform ethanol methylal 1:1:1 (twice the original volume) and refluxed for four hours. A further extraction of the residue with chloroform ethanol methylal yielded no cholesterol and was discarded. As before, the pooled extracts were evaporated to small volume and re-extracted four times with petroleum ether (200, 200,100 and 100 ml.)

Small portions of serum were removed in each case for total and free cholesterol estimation.

The protein residues from the extraction processes were incubated overnight with 100 ml. of 25% w/v potassium hydroxide solution, by which time the protein was dissolved. The excess alkali was neutralised with hydrochloric acid and the mixture extracted repeatedly with petroleum ether. The combined petroleum ether extracts were washed with water, dried over sodium sulphate, and the solvent distilled off. Quantitative Lieberman-Burchard reactions were performed on the residues and showed only traces of cholesterol (0.05-0.60 mgm. total).

(2) Chromatographic Separation of Cholesterol Esters

Chromatography on alumina was attempted first. The initial experiments were based on the work of Kerr and Bauld (1953). Using 4 mm. columns of alumina activated to grade 11 (Brockmann and Schodder 1941) it was found possible to separate ester and free cholesterol by elution with

petroleum ether (B.P. 60°-80°) and benzene respectively, and quantitative measurement by the Lieberman Burchard reaction gave results which agreed closely with digitonin precipitation. When larger columns were used, however, it was found difficult to elute the ester fraction and use of solvents higher in the elutotropic series gave poor separations. Alumina of lower activity was then used and with grade IV, quantitative yields of ester as measured by Lieberman Burchard were again obtained. Upon hydrolysis, however, this fraction yielded no acids and it was suspected that hydrolysis was occurring on the strongly alkaline columns in use (this phenomenon has already been noted in connection with triglycerides by Borgström (1942 a & b)). Columns were then prepared from Wöelm neutral alumina (L. Light & Co.) also activated to grade IV and with this material, apparently quantitative yields of ester were obtained both by gravimetric and colorimetric estimation; upon hydrolysis, however, only about one half of the expected yield of acids was obtained.

At this juncture a method of separating blood lipids on columns of silicic acid was published (Fillerup and Mead, 1953). This method was investigated, firstly using silicic acid (British Drug Houses) which gave poor separation and later with silicic acid chromatographic (L. Light & Co.). The latter gave satisfactory separation of cholesterol ester from a representative artificial mixture.

The results reported here were obtained by the use of silicic acid columns prepared as follows. The silicic acid

was boiled with two or three volumes of methanol. The methanol was decanted and replaced twice. This process was repeated using acetone and finally again with ether. The ether was allowed to evaporate and the dry powder was heated to 100°C. overnight. It was allowed to cool partially in the oven, and then transferred rapidly to airtight containers for storage. When required the silica was weighed quickly in a dry vessel on a coarse balance and immediately flooded with petroleum ether (B.P. 40°-60°) If these precautions were not observed, the silica absorbed sufficient moisture in a short time to become hot and presumably lost much of its adsorptive capacity. The silica was then transferred to the column as a slurry with petroleum ether, using a large bulb pipette with no tip and a rubber ball teat for the transfer; the silica was not tamped but was allowed to settle by gravity whilst petroleum ether flowed through the column. Commercial columns were used, (Quickfit and Quartz Ltd.) 18 mm. bore with a coarse sintered disc at the bottom; 30 g. of silica was normally used giving a column 30 cm. in length.

Mixtures of lipids up to 0.9 g. were found to separate satisfactorily on these columns. The lipids were added in solution in the minimum volume of petroleum ether (B.P. 40°-60°) and followed by 300 ml. of petroleum ether (B.P. 40°-60°). Petroleum ether containing 1% of peroxide free ether was then used to elute cholesterol esters. 50 ml. fractions were collected and a 1 ml. portion of each was

evaporated to dryness and tested by the Lieberman Burchard reaction. Elution was continued until the reaction was negative.

In the case of Sample 11 elution was continued further, 3% ether in petroleum ether, 10% ether in petroleum ether and ether methanol equal parts were used successively to elute the triglycerides, the free alcohol and the phospholipids. In this experiment three 1 ml. portions were removed from each 50 ml. fraction and were examined for cholesterol, glycerol and phosphorus respectively. In the other three experiments glycerol and phosphorus analyses were not performed on the individual fractions but a portion of the combined Lieberman Burchard positive material was removed for these tests. The techniques used for glycerol and phosphorus estimation are given in the section dealing with analytical procedures.

(3) Hydrolysis

The cholesterol ester fraction was evaporated to dryness, weighed and transferred in the minimum quantity of peroxide free ether to a semi-micro reflux apparatus, and 10 ml. of 20% sodium ethoxide were added; nitrogen was supplied to the flask by a glass capillary which ran the length of the condenser. The mixture was refluxed for three hours, the condenser was then removed leaving the nitrogen tube in situ and the alcohol allowed to evaporate. The residue was shaken with 10 ml. each of water and peroxide free ether under nitrogen and when dissolved, transferred to a separating funnel. The ether layer was washed twice with water (10 ml.) and each aqueous sample was in turn washed

with ether. The aqueous samples were pooled, made just acid to congo red with 10 per cent hydrochloric acid, and extracted three times with 30, 20, and 10 ml. of ether. The ether extracts were pooled and washed once with 20 ml. of water. Troublesome emulsions were encountered, and the soaps were sometimes retained in the ether layer; for this reason petroleum ether (B.P. 40°-60°) was used in the later experiments.

(4) Reversed Phase Chromatography of Fatty Acids

Columns were first prepared in the manner described by Howard and Martin (1950). Kieselguhr (Hyflo-super-cel Johns Mandeville) was rendered non wettable by exposure to the vapour of dichloro-dimethyl silane in a vacuum desiccator for two hours. It was then washed with methanol until free from acid and dried in the hot air oven at 100°C.

Medicinal paraffin was equilibrated with 60% acetone/water by shaking in a separating funnel and then mixed with the siliconed kieselguhr in the proportion of 9 g. kieselguhr to 8 ml. of equilibrated paraffin. The mixture was stirred in a beaker until homogeneous and then made into a slurry with the 60% acetone layer from the equilibration mixture. The slurry was transferred to the column with a large bulb pipette without a tip, fitted with a large rubber ball teat (as was used in the preparation of the silicic acid columns.

Columns were of 18 mm. bore hard glass, manufactured by Quickfit and Quartz Ltd. and were packed with 25-35 g. of paraffin kieselguhr mixture. Temperature was maintained

at 33°-36°C. by a thermostatically controlled hot air jacket. This was left on during the night to prevent acids crystallizing out on the column (Howard and Martin, 1950).

It was found impossible to pack these columns with a plunger as described by Howard and Martin, and the stationary phase was allowed to settle as solvent was run through. Fatty acids were added to the column in the minimum volume of 60% acetone which was allowed to run very slowly into the column, followed by 45% aqueous acetone. Acids were eluted with increasing strengths of acetone equilibrated with liquid paraffin.

The eluate was collected in 5 ml. fractions with a home-made fraction separater, details of which are given in the subsequant section. The acids were titrated with N/50 tetramethyl ammonium hydroxide in 50% ethanol in a Conway microburette using 0.1% bromothymol blue. A stream of nitrogen was used for stirring.

These columns did not behave satisfactorily; flow rates were extremely slow (5 ml. per hour or less) and the stationary phase frequently collapsed and shed liquid paraffin when the mixture of acids was added. These phenomena appeared to be due to the small particle size of the batches of kieselguhr now available. More recent methods of preparing medicinal paraffin columns (Silk and Hahn, 1954) (Wittenberg, 1957) include procedures for removing the fines before treating the kieselguhr with silicone.

New columns were therefore prepared as described by Silk

and Hahn. Hyflo-super-cel (5 lb.) was stirred with three gallons of tap water and allowed to stand. After one hour the supernatant including fines was decanted. The process was repeated on the sediment once and the final product dried in the hot air oven at 100°C. The dried kieselguhr was treated with dichloro-dimethyl silane as before and washed free from acid with methanol. 93 g. kieselguhr was then mixed with 75 ml. medicinal paraffin in 500 ml. pure ethyl-ether (British Drug Houses Ltd. Micro Analytical Reagent grade). The ether was distilled off under reduced pressure with mechanical stirring, andthe dry powder was maintained at 60°C under reduced pressure for four hours.

Columns were packed with 25-35 g. of stationary phase, the latter being suspended in 60% acetone equilibrated with liquid paraffin. The mixture was warmed on the steam bath to about 50°C. to expel dissolved air and then was transferred to the column with the bulb pipette as already described. Firm packing was effected by lowering a wad of cotton wool on to the surface of the kieselguhr and forcing this down with a stainless steel plunger. Columns did not give good separation unless firmly packed. As before columns were maintained at 33°-36°C.

Fatty acids in quantities from 10-65 mg. were added to the column in the minimum quantity of pure acetone necessary for complete solution. As soon as the acetone was absorbed into the top of the column, 45% aqueous acetone equilibrated with liquid paraffin was added and the column was allowed to run very slowly for 2-3 hours. Solution in small volumes

of pure acetone was effective for unsaturated acids, but it was found that saturated acids could not be dissolved in such small volumes. The technique devised by Silk and Hahn was therefore adopted. The acids were dissolved in liquid paraffin, 0.2 ml. to each 7 mg. acids, gentle warming hastening solution. This mixture was dissolved in 15 ml. of ether and 0.24 g. of siliconed kieselguhr added. The ether was evaporated under reduced pressure in a stream of nitrogen and a temperature of 60°C was maintained for four hours. The fatty acid, paraffin, kieselguhr mixture was suspended in 40% acetone and transferred to the column.

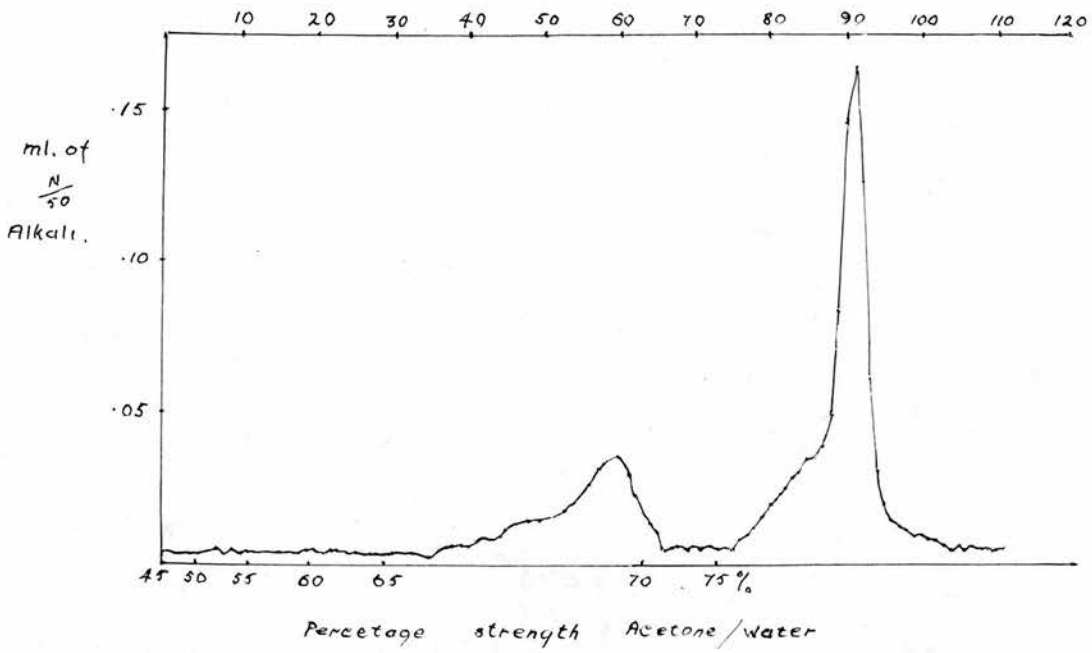
Columns were operated at a flow rate of 30-40 ml. per hour. Elution was carried out using ascending strengths of acetone/water, commencing at 45% and increasing to 80%. The increments were usually by 5% steps but in Experiment 11, 2½% steps were used. No advantage was found in this practice and 5% steps were used in all subsequent experiments. About 1.25 column free space volumes of each successive strength of acetone was run through the column and if no acid appeared, the next strength was added. If, however, an acid was eluted, the acetone strength was not increased until the flow of acid from the column was diminishing.

The behaviour of these columns was examined by adding known quantities of purified acids of chain lengths from C6-C20 (all even numbers of carbon atoms). All acids of chain length below and including C8 were eluted with 35-40%

FIGURE 1.

Reversed Phase Chromatography

of Palmitic and Stearic Acid.



3 mg. Palmitic acid and 6 mg. stearic acid added to column. Recovery, by titration 2·4 mg. Palmitic and 5·0 mg stearic acids. Total Recovery 82%

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acetone, C10 acids with 45%, C12 with 50%, C14 with 55%, C16 with 65%, C18 with 70 or 75% and C20 with 80%. Unsaturated acids were eluted with lower strengths of acetone than wouldbe expected from their chain length, the effect of one double bond being to make the acid behave as if its chain were two carbon atoms shorter than it actually was. A representative graph showing the separation of pure palmitic and stearic acids by this technique can be seen in Figure 1.

Recoveries of acids from these columns as measured by titration were usually of the order of 80-90% though recoveries as low as 60% were obtained when quantities under a total of 4 mg. were examined. Silk and Hahn (1954) noted similar apparent losses and concluded that the anomaly lay in the end point of the titration. Despite these losses, quantities of acids recovered from mixtures remained proportional to each other.

Further small anomalies were noted in practice. Firstly, the application of very large volumes of a given strength of acetone resulted finally in the slow elution of acids which normally require the next higher strength, the acid being eluted as a low plateau instead of a sharp peak. In addition, with heavy loading, especially of saturated acids, the longest chain acids were sometimes incompletely eluted by their appropriate solvent and the residual portion produced a separate peak with the next increment of solvent strength.

None of these difficulties was encountered with

unsaturated acids but with fully saturated material it was found desirable to keep the loading below a total of 10 mg. of mixed acids.

The acids recovered from hydrolysis of cholesterol esters were treated on the columns as described and the eluted fractions collected, titrated with standard alkali and the results plotted as a graph. The fractions forming individual peaks were pooled, evaporated to small volume in an atmosphere of nitrogen, acidified and extracted with petroleum ether. After drying overnight over anhydrous sodium sulphate the extracts were evaporated to dryness and then hydrogenated. The acids recovered after hydrogenation were again examined by reversed phase chromatography.

Molar proportions of acids forming each peak were calculated from the sum of the titration figures after subtraction of the blank titration for the appropriate strength of acetone water.

In Experiments III and IV iodine values were estimated both on the total acids recovered from hydrolysis and also on those recovered from the larger peaks of the first chromatographic separation before hydrogenation.

In Experiments II and III, a portion of the acids recovered from hydrolysis was oxidised by the modified method of Bertram as used by Crombie, Comber and Boatman (1954). The acids recovered after this treatment consist of unchanged saturated acids from ~~in~~ the original mixture, plus the oxidation products of such unsaturated acids as were originally

present. When such a mixture is treated chromatographically, the oxidised acids are all eluted with 40-45% acetone whilst the saturated acids appear in their expected positions.

The fatty acids recovered in Experiment 111 were subjected to alkaline isomerisation by the micromethod of Holman (1957), and examined on a "Unicam" spectrophotometer; details of the method used are to be found in the subsequent section.

11. Analytical Procedures

Reagents:

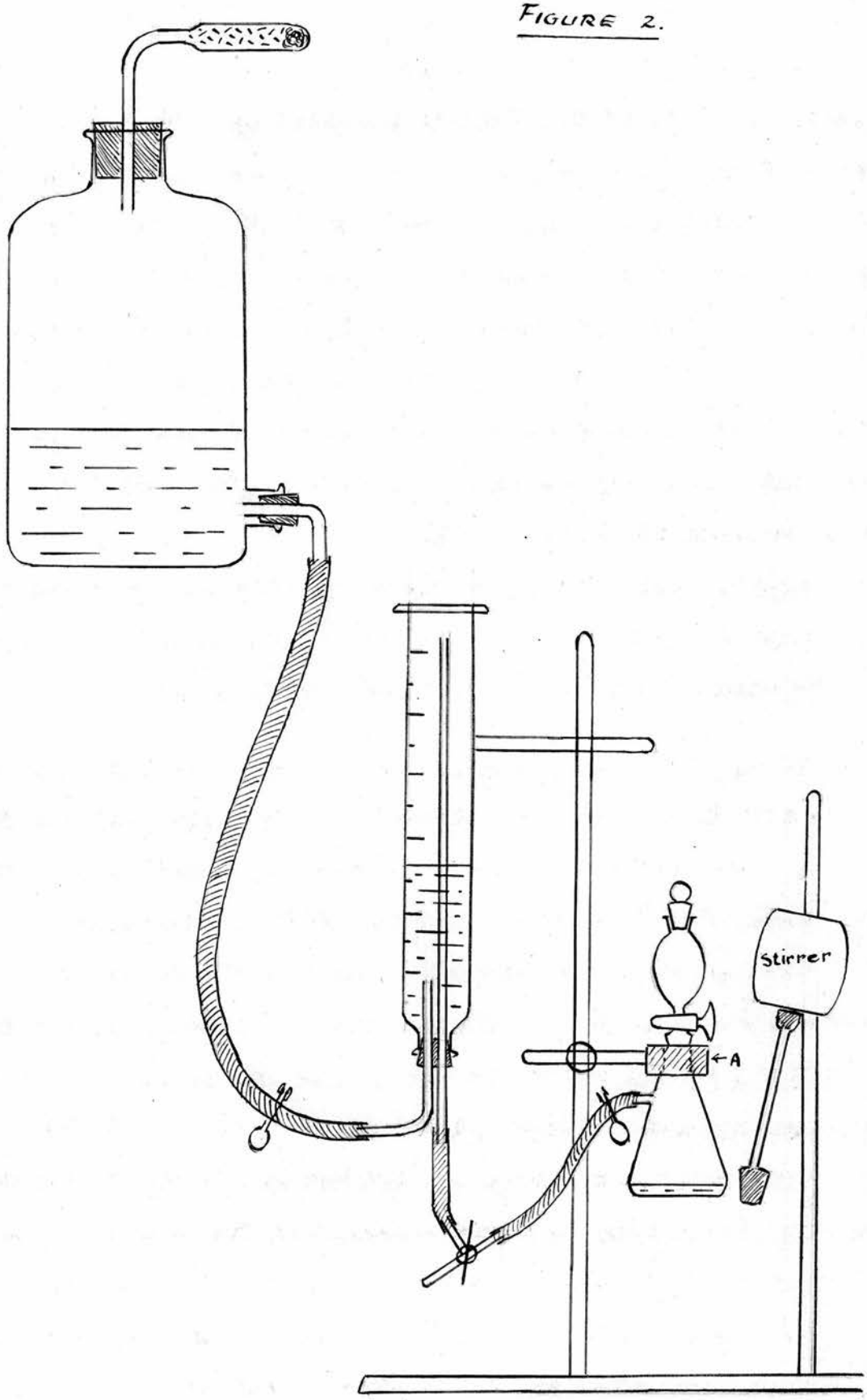
All solvents were of analytical reagent grade except for the ether which was of Micro Analytical grade (British Drug Houses). They were not further purified. The acetone was very slightly acid and produced a small blank which was allowed for in calculating the recoveries of acids.

The water was of high purity, obtained by passing distilled water down a column of ion exchange resin (Amberlite M.B.3, British Drug Houses).

The sodium, the tetramethyl ammonium hydroxide, the decolourising charcoal and the palladium chloride were of laboratory reagent grade. All other reagents were of analytical grade.

Specimens of purified palmitic, lauric, myristic and oleic acids were generously donated by Mr. R. F. Nunn,

FIGURE 2.



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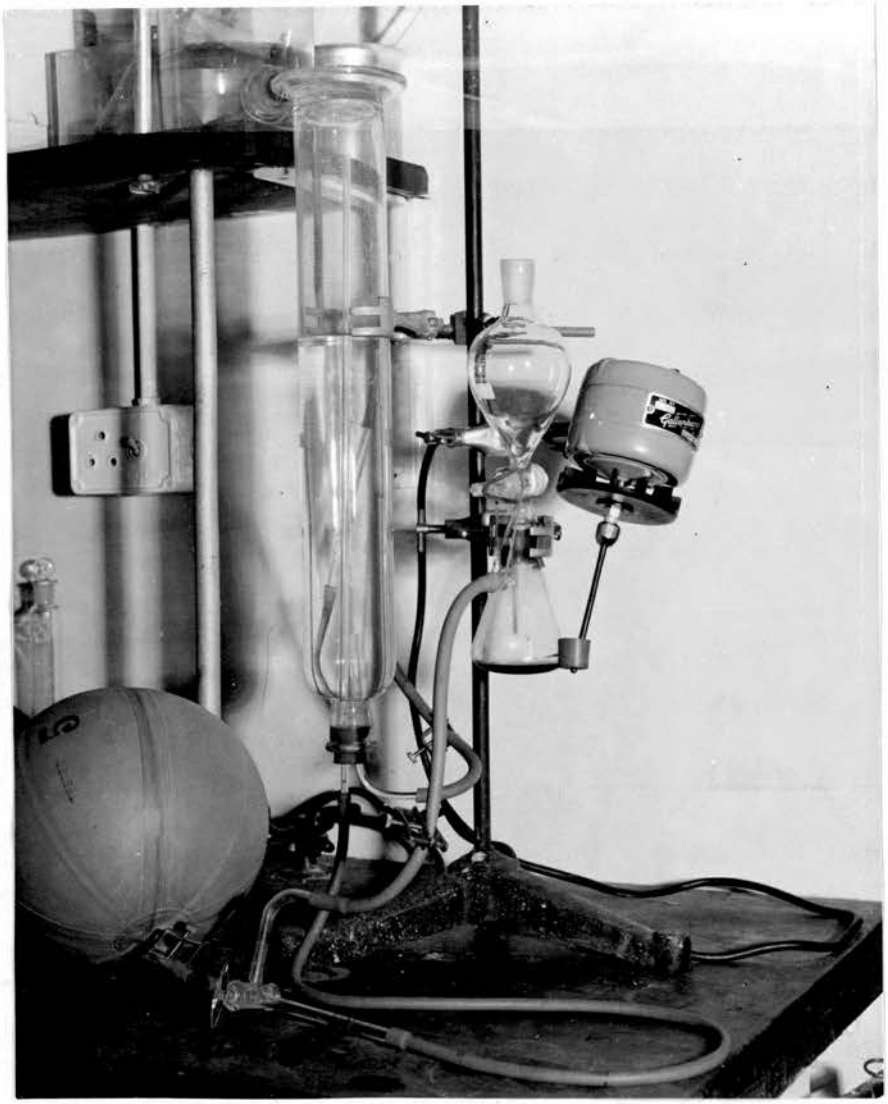
B.Sc., F.R.I.C. of St. Francis Hospital and the Royal Sussex County Hospital, Brighton. A specimen of pure linoleic acid was kindly provided by Unilever Research Laboratories of Port Sunlight, Cheshire. Stearic acid was obtained by the hydrogenation of elaidic acid and proved to be of high purity when examined chromatographically. This method of preparation was found to be necessary as the commercial stearic acid available was contaminated with large amounts of palmitic acid.

Palladinised Charcoal - for the catalytic reduction of unsaturated fatty acids was prepared from palladium chloride by the technique described by Vogel (1948).

15 g. of charcoal was heated on the steam bath for three hours with 10 ml. of concentrated hydrochloric acid and 300 ml. water. It was then washed with water by decantation until acid free, filtered, and dried at 100°C. Palladium chloride 1.0 g. was dissolved in 1.5 ml. of concentrated hydrochloric acid and 10 ml. of water with heating, and added to 100 ml. of 35% sodium acetate, this was placed in the hydrogenation apparatus described below, and 11.5 g. of the purified charcoal was added. Hydrogenation was continued for five hours when hydrogen consumption had ceased. The catalyst was collected on a Buchner funnel with several thicknesses of Whatman No. 1 filter paper, washed five times with distilled water and dried over silica gel in vacuo.

The Hydrogenation apparatus was also arranged as

PLATE I



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described by Vogel (1948), see Figure 2 and Plate 1.

The inverted, stoppered measuring cylinder formed the hydrogen reservoir and was connected by a long stand pipe to the side arm of a Buchner flask fitted with a tap funnel. A side arm in the connecting tube permitted the admission of fresh supplies of hydrogen. Positive pressure was provided by an aspirator jar of water 18" above the apparatus and connected to the lower end of the measuring cylinder. The Buchner flask was mounted in a soft rubber block "A" and an eccentric rubber bung driven by a stirrer was arranged to strike the flask and thereby agitate the contents. The rubber football bladder visible in the photograph was used to convey small volumes of hydrogen to the apparatus.

Samples of acid to be hydrogenated, (of the order of 20 mg.) were introduced into the flask in solution in 5 ml. of ethyl acetate, 50 mg. of catalyst was introduced, care being taken to see that it was covered by solvent. The tap funnel was placed in position and about 400 ml. of hydrogen was blown through the apparatus, the tap on the funnel being intermittently closed. More hydrogen was admitted to lower the water level in the reservoir to the zero mark which gave a pressure of eighteen inches water gauge.

The relatively large proportion of catalyst was found to be necessary for the reaction to take place in a reasonable length of time, and it was also found necessary to continue for at least seventy-two hours to ensure complete hydrogenation.

PLATE II

PLATE III

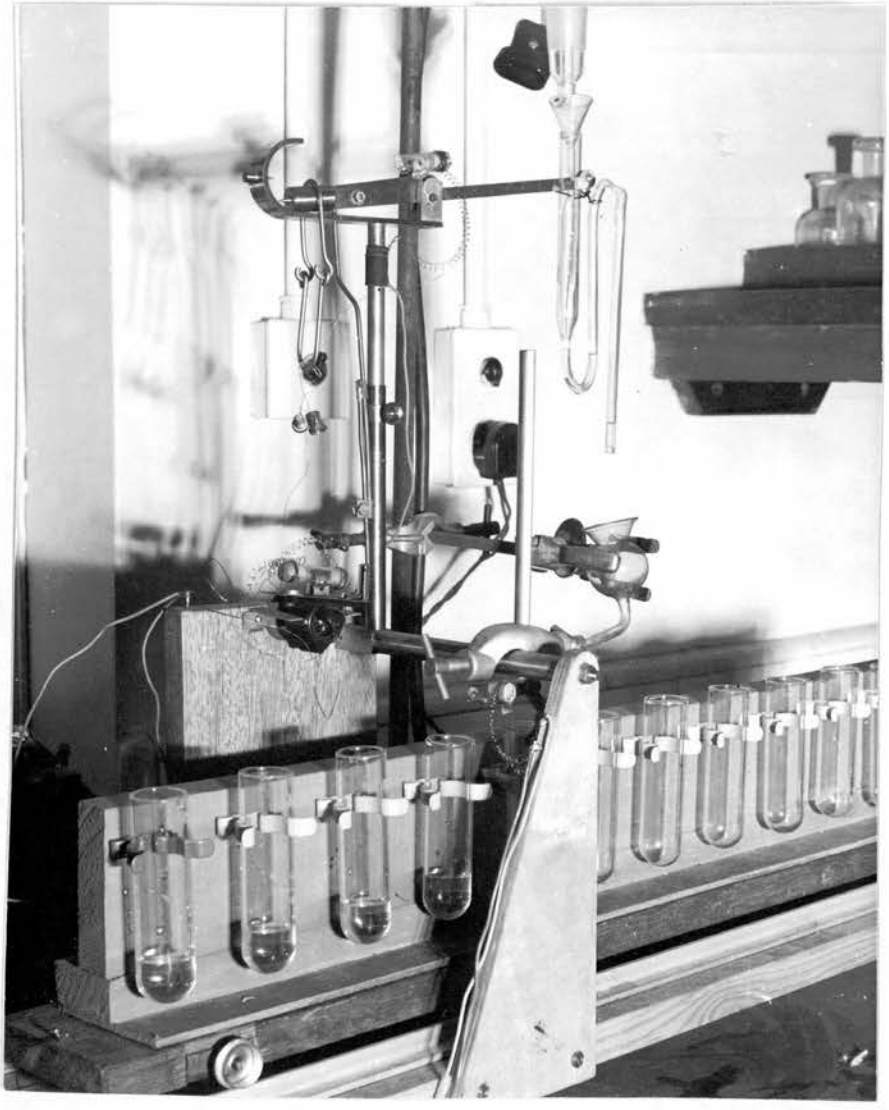

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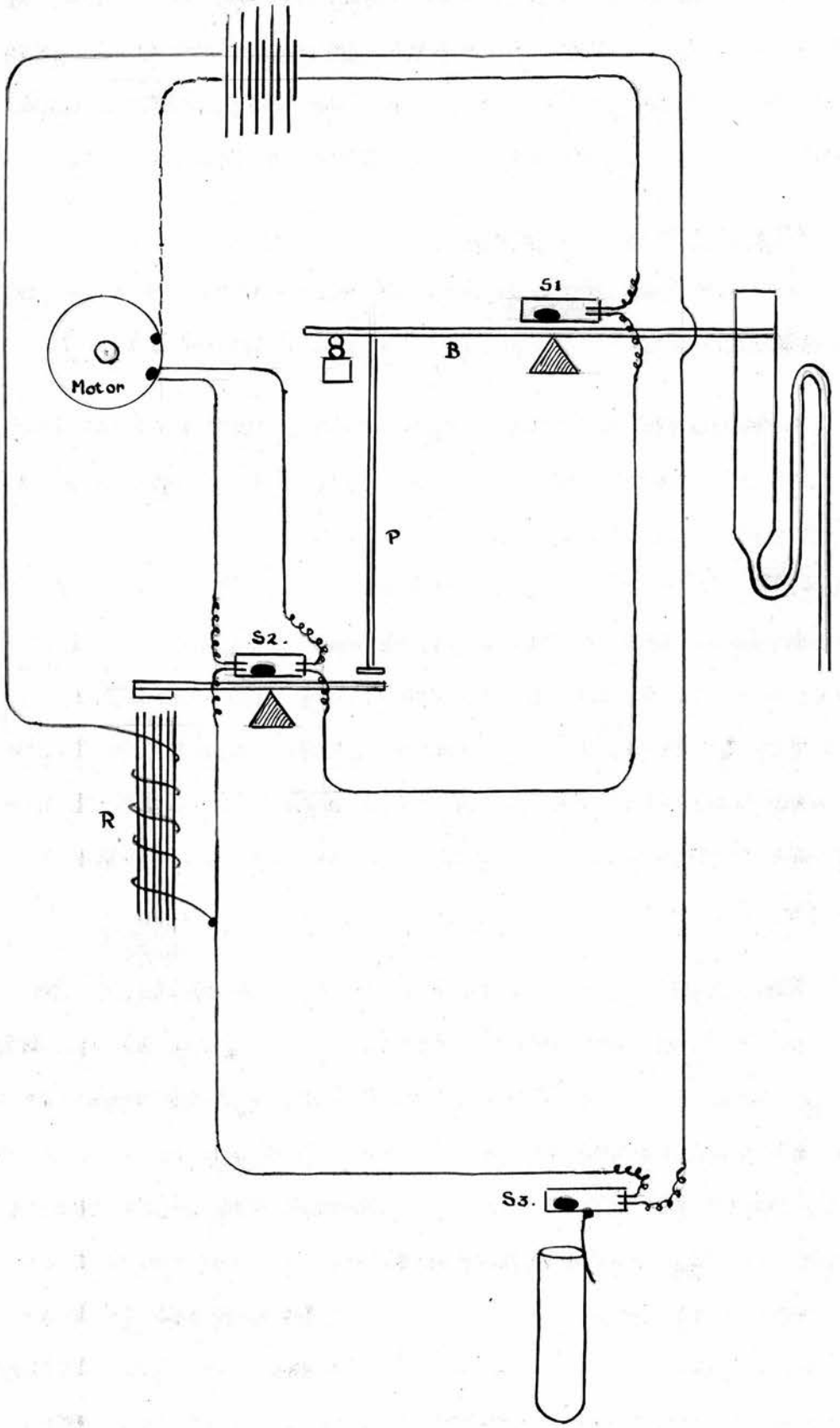
FIGURE 3.



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The degree of reduction achieved was estimated by the iodine number of the recovered sample, a value of less than five, (the limit of accuracy of the micro method used) being accepted as evidence of complete saturation.

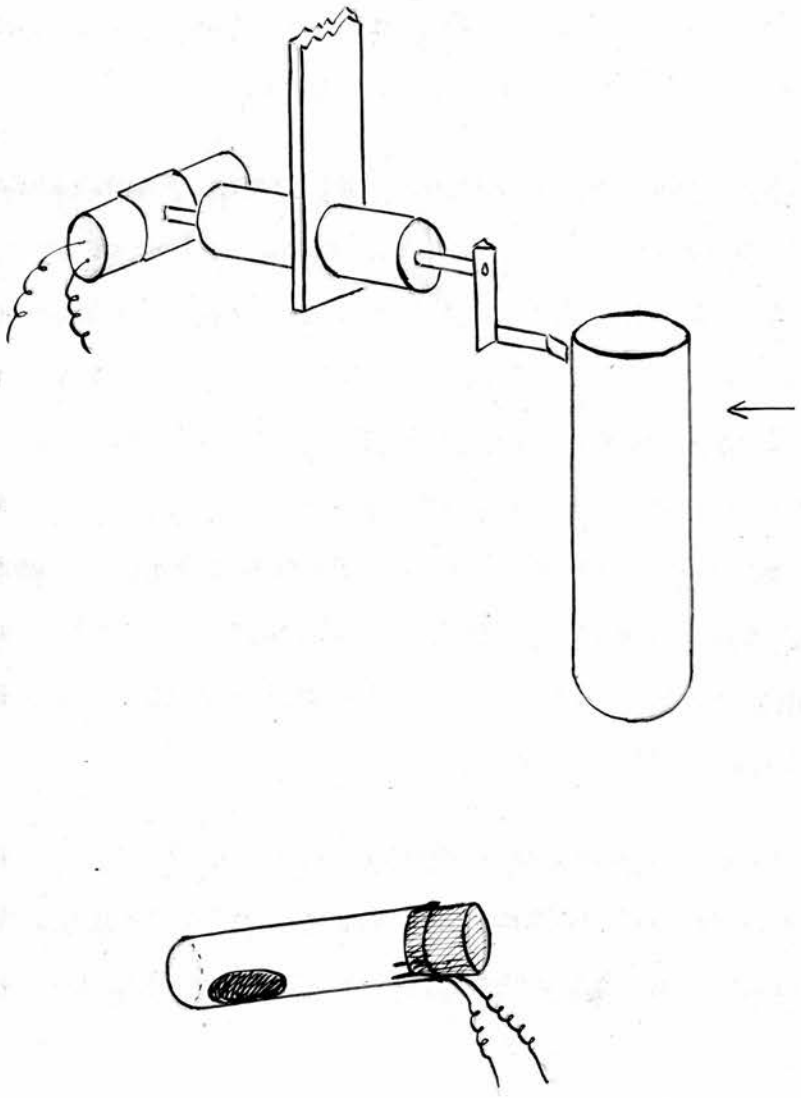
The Fraction Separator:

The general arrangement of this device can be seen in the photographs (Plates 11, 111 and Figures 3 & 4)

A carriage bearing twelve 30 ml. pyrex tubes is drawn along a straight tramway by a winding gear and enless cord driven by a miniature electric motor. Each tube as it comes in contact with a pivoted arm operates a mercury switch which opens the motor circuit and arrests the tube under the outlet of the siphon. The siphon of 5 ml. capacity is mounted on a counterpoised arm which bears another mercury switch; whenthe siphon is half filled, the arm overbalances, the switch closes and starts the motor.

The circuit, shown in Figure 3, was modified from that of Brimley and Snow (1950). The cycle of operations is as follows:- as the siphon fills, the pivoted arm B. overbalances to the right and switch S1. closes; current flows round the green circuit through the right hand end of switch S2. andthe motor starts. As the centrifuge tube is drawn away from switch S3. this is allowed to tilt to a closed position; this allows current to flow through the relay R. which causes switch S2. to tilt to the left. The green circuit is now broken and the left-hand end of switch

FIGURE 4



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S2. closes allowing current to continue to flow to the motor. When a fresh tube is drawn into position switch S3. is opened and the action ceases. The siphon continues to fill and finally empties into the fresh container. The arm B. now overbalances to the left, Switch S1. opens and the push rod P. tilts switch S2. back to the right hand position. The cycle is now ready to start again.

It was found that commercial mercury switches were too heavy and required too great an angle of tilt to operate satisfactorily on this instrument; the switches finally used were constructed from thin-walled flat bottomed test tubes of 8 mm. bore; about 1.0 g. of mercury was placed within and two platinum wires placed close together under the cork so that their tips projected 2 mm. on the inside. The corks were waxed in and no attempt was made to evacuate the trapped air. No difficulty has arisen from oxidation at the low potential used.

The entire apparatus operates at six volts from a large capacity dry battery. The general construction of the switches and the trip gear of switch S3. are shown in Figure 4.

The Column Heater:

Columns used in earlier experiments were wound externally with about 2 metres of wire from a 500 watt element from a domestic iron. About 20 volts potential was sufficient to heat the columns to 35°C. and normal control of temperature was obtained with a Variac transformer. The heating element was covered with asbestos string and a

thick layer of cotton wool. Local overheating which disrupted the column occurred with some frequency, and it was not safe to continue heating the columns overnight.

An air jacket of tinplate was therefore constructed; the ends were closed with wooden plugs drilled to admit the column, a thermometer and an oven type thermostat. A perspex window in the upper third of the jacket allowed inspection of the surface of the column packing and the lower two thirds was wound externally with two 50 watt isotapes and lagged with cotton wool. The isotapes were connected in series and a 120 volts potential was applied from the Variac transformer. The thermostat required a 3°C. change of temperature to actuate the switch so that in practice, column temperatures ranged from 33°C-36°C. but were constant within those limits.

The Oxidation Process:

The Bertram process of oxidation (treatment with alkaline permanganate at room temperature) as employed by Crombie, Comber and Boatman (1955) was used without modification except that the quantities of acid available were much smaller, i.e. about 15 mg. instead of the 100 mg. used by the original authors.

The sample of fatty acid mixture was heated with 1 ml. 0.5 N. potassium hydroxide in methanol and 5 ml. of water. After evaporation of the methanol and cooling, 0.2 ml. of 50% (W/V) aqueous KOH and 15 ml. of 5% (W/V) potassium permanganate solution were added, andthe mixture set aside

at room temperature for 16-18 hours. At the end of this time, 2 ml. of 50% (V/V) sulphuric acid were added, followed by a concentrated solution of sodium metabisulphite until the permanganate solution was just decolourised. The mixture was then warmed to just below boiling point. After cooling, the fatty acids were extracted three times with 5 ml. of petroleum ether (B.P. 40°-60°) and the combined extracts washed four times with 15 ml. portions of distilled water. The washed extract was dried over anhydrous sodium sulphate and evaporated to dryness. The recovered acids were then examined chromatographically.

In the original experiments, the completeness of oxidation was controlled by iodine number estimations. In the experiments described here the iodine numbers fell to about 20 after the first 18 hours and remained at that level or increased after several days oxidation. In Experiment 111, after 18 hours oxidation, the iodine numbers by Yasuda's method were found to be 22.0. After 48 hours oxidation they were found to be 29.5. The determination was then repeated using Hubl's method which gave an iodine number of 55.0. Since the iodine numbers could not be relied upon as a guide to the completeness of oxidation, the acids were oxidised for 48 hours instead of the 18 hours advocated in the original work.

Phosphorus:

Phosphorus analyses were performed on fractions recovered from the silicic acid columns, by the method of Fiske and Subbarow (1925) after wet ashing. In Experiment 11

a 1.0 ml. portion from every 50 ml. fraction was examined, but in the other experiments 5 ml. portions from the combined cholesterol ester fractions alone were examined.

In each case, the solvent was evaporated on the steam bath and the residue was digested with 1.5 ml. of 10 N sulphuric acid over a small flame. If charring occurred, a drop of 30 volume hydrogen peroxide was added and heating continued. This process was continued until the solution was colourless. The solution was made up to 15 ml. with water, then 2.5 ml. of 2.5% (W/V) ammonium molybdate and 1.0 ml. of 1:2:4 amino-naphtholsulphonic acid were added and the volume made up to 25 ml. with water. A blank tube and a solution containing 0.08 mg. of inorganic phosphorus were similarly treated. Colour was allowed to develop for ten minutes and the absorption was measured in an E.E.L. colorimeter (Evans Electroselenium Co.) with an Ilford 205 filter.

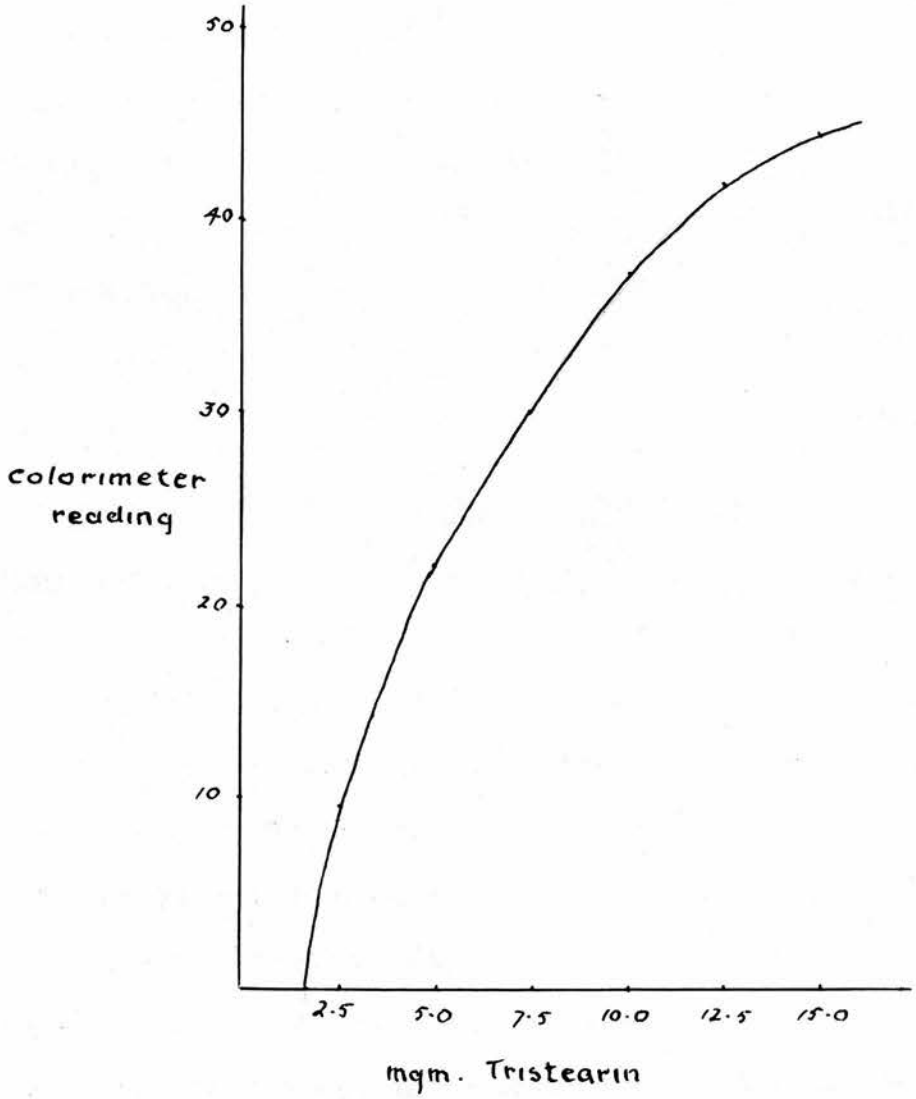
Glycerol:

(Lambert and Neish, 1950) As in the case of the phosphorus analyses glycerol was estimated in a 1 ml. aliquot in Experiment 11 and in a 5 ml. aliquot from the combined cholesterol ester bearing fractions in the other experiments.

After evaporation of the solvent, 1 ml. ethanol and 1 ml. of 50% (W/V) potassium hydroxide were added, and the mixture heated for one hour on the Boiling water bath. The excess of alkali was neutralised with 10 N. sulphuric acid and a further 1.0 ml. of this acid added. 5 ml. of

FIGURE 5

Glycerol Analyses



0.1 M. sodium periodate were added and after mixing, the mixture was set aside for exactly 5 minutes. 5 ml. of 1.0 M. sodium arsenite were then added and the mixture set aside for a further 10 minutes and the volume was then made up to 100 ml. with water. 1.0 ml. of this was pipetted into a pyrex boiling tube and 10 ml. 1% (W/V) chromotropic acid was added. The tubes were heated for 30 minutes, cooled and absorption measured in the E.E.L. colorimeter using an Ilford 626 filter. A blank tube, and 1 ml. of a standard containing 1 mg. of glycerol in water were similarly treated.

When this technique was initiated its reliability was investigated. A range of samples of glycerol tristearate ranging from 2.5 mg. to 15 mg. were examined. The colorimeter readings expressed graphically are shown in Figure 5. The recoveries of glycerol calculated from these results ranged from 96.5% in the case of the 2.5 mg. sample to 83% in the 15 mg. sample. A sample of 11 mg. of triolein gave a recovery of glycerol of 103%.

The Quantitative Lieberman-Burchard Reaction:

This analysis was carried out on 1 ml. portions of the eluate from the silica columns. The solvent from each sample was evaporated on the steam bath and after cooling, 5 ml. of chloroform were added, followed by 2 ml. of a freshly prepared and cooled mixture of acetic anhydride 10 parts and concentrated sulphuric acid one part. The samples were left in the dark for thirty minutes at room temperature for the colour to develop and their absorption measured in the E.E.L. Colorimeter using the Ilford 205 (red) filter.

A standard containing 0.4 mg. of cholesterol and a blank of 5 ml. of chloroform were similarly treated. This test was performed on all the fractions collected in all four experiments and provided a guide for the location of the cholesterol esters.

Estimation of Esterified and Free Cholesterol:

Esterfied and free cholesterol were estimated on all samples of serum before extraction was begun, by a modification of the method of Obermer and Milton (Milton & Waters, 1955).

4 ml. of serum were pipetted, dropwise, into 75 ml. of acetone ethanol mixture 1:1 in a 100 ml. volumetric flask, heated to boiling on the steam bath and allowed to cool to room temperature, then made up to 100 ml. and filtered.

Estimation of free cholesterol:- 10 ml. of extract in a 30 ml. pyrex centrifuge tube were evaporated to dryness with 0.5 ml. of 1.0% digitonin in 50% ethanol. 2 ml. of water were added, the contents of the tube heated to boiling, andthe colloidal solution formed broken up by addition of 4 ml. of acetone. One drop of 4% aluminium chloride was added and after mixing, one drop of strong ammonia was added. The aluminium hydroxide formed was centrifuged hard and packed down with the cholesterol digitonide. The supernatant was decanted and the aluminium hydroxide dissolved in one drop of 30% hydrochloric acid. 4 ml. of acetone were then added and the whole centrifuged hard for five minutes. The supernatant was removed and

the precipitate again washed with 2 ml. of acetone. Finally the precipitate was washed with 3 ml. of ether, centrifuged, decanted and the residual ether allowed to evaporate spontaneously. The precipitate was then dissolved in glacial acetic acid 2 ml., the tube being heated just to boiling, over a micro-burner. After cooling, 2 ml. of freshly prepared and cooled acetic anhydride-sulphuric acid mixture 10:1 were added and the tubes left in the dark at room temperature for one hour for the colour to develop. Absorption was measured in the E.E.L. Colorimeter with an Ilford 205 filter.

Estimation of Total Cholesterol:- 5 ml. of acetone alcohol extract were refluxed for 30 minutes with 5 ml. of sodium ethylate (5 g. of sodium in 100 ml. ethanol). The bulk of the alcohol was evaporated and 15 ml. of petroleum ether (B.P. 40°-60°) and 2 ml. of water were added and the contents thoroughly shaken. The petroleum ether was filtered into a clean flask using a pasteur pipette for the transfer. The residue was again extracted twice with 10 ml. of petroleum ether, and after addition of 0.5 ml. of 1% digitonin solution, the petroleum ether was distilled off. 2 ml. of water were added and heated to boiling; then, after addition of 4 ml. of acetone, the contents were transferred to a centrifuge tube, the flask being rinsed twice with 2 ml. of acetone. Washing and colour development were then continued as for free cholesterol.

A standard of 0.5 mg. of cholesterol in 5 ml. of acetone ethanol and a blank of 5 ml. of acetone ethenol were treated along with the free cholesterol.

The original method employs the reaction between an acyl chloride and cholesterol in the presence of zinc chloride. This was abandoned because the O-nitrobenzoyl chloride recommended by the authors is dangerous to prepare and of the alternative acid chlorides available, benzoyl chloride gave a precipitate (probably of benzoic acid), whilst acetyl chloride gave variable results.

It was found difficult to remove supernatants by decantation without dislodging the precipitate; they were therefore removed with the aid of a Pasteur pipette drawn out to a fine tip and bent over; gentle suction was applied with the filter pump.

All estimations were performed in duplicate and the average of colorimeter readings was used for the calculations.

Iodine Numbers:

The micro-method of Yasuda (1931) was used. This method employs the Rosenmund and Kuhnhenn halogenating reagent prepared as follows: 16 g. of purified pyridine and 20 g. of concentrated sulphuric acid were measured into separate flasks each containing about 40 ml. of glacial acetic acid and the two were then combined. 16 g. of bromine dissolved in 40 ml. of glacial acetic acid were added to the mixture and the whole diluted to a final volume of 2 litres with glacial acetic acid.

The sample of lipid to be halogenated (2-5 mg.) was dissolved in 2 ml. of chloroform in a glass stoppered flask, in duplicate if sufficient material was available. Two

blank flasks containing 2 ml. of chloroform were also prepared. 5 ml. of the pyridine sulphate dibromide, diluted with glacial acetic acid so that the bromine was about 0.02 normal, were added to each flask. The flasks were stoppered and left at room temperature for fifteen minutes; 2 ml. of 2% potassium iodide and a few drops of sodium starch glycollate were added to each flask and the liberated iodine was titrated with 0.02 N. sodium thiosulphate. The thiosulphate solution was standardised at each test against 0.1 N. sodium iodate. A control sample of 4 mg. of pure cholesterol in chloroform was included with each batch of unknowns.

Alkaline Isomerisation:

Alkaline isomerisation was performed on the acids recovered in Experiment 111. The micromethod of Holman (1957) was employed and the constants quoted by this author used in the calculation.

Apparatus:

The heater block was constructed by Mr. D. Budgeon of St. Francis Hospital and was modified from that described by Lips and Tessier (1949). It consisted of a cylindrical block of aluminium 6" long by 3" diameter with four $\frac{7}{8}$" holes drilled along its length and one $\frac{1}{4}$" hole drilled along its axis. The centre hole contained a thermometer, one of the large holes contained a mercury thermoregulator, whilst the remaining three holes contained Folins blood sugar tubes, calibrated to contain 5 ml. and fitted with

aluminium lids. The block was wrapped in asbestos paper and wound with three 500 watt spiral elements; two of these elements were in series and were connected across the main tappings of a variac transformer, the third was connected through a post office type relay to the 150 volt tapping of the Variac. The post office relay was connected to the thermoregulator in such a manner that when the mercury rose and made contact, the relay opened. The block was packed in expanded mica in a tinplate container 9" in diameter by 10" long. A booster switch was provided to place all three elements in parallel and permit the temperature to be raised quickly. To improve conduction of heat, the space around the thermoregulator was packed with copper filings whilst the spaces around the reaction tubes were filled with silicone oil (Midland Silicone 550). The apparatus maintains a temperature of 180°C ±0.2°C.

During isomerisation, nitrogen was fed through fine polythene tubing to glass capillaries which pierce the lids of the Folin's tubes.

The reagent was prepared by heating 100 ml. ethylene glycol to 190°C. and allowing it to cool to 150°C. 28 g. of 85% aqueous potassium hydroxide was poured carefully in, the solution reheated to 190°C. for ten minutes, and then allowed to cool. The whole process was performed under nitrogen. Samples of the alkaline glycol were titrated and the concentration adjusted to 21% ± 0.1%.

1.1 g. of reagent was weighed into theFolin's tubes on a coarse balance and 0.25 - 0.5 mg. of fatty acids added in solution of 1 ml. ethanol. After mixing, the tubes were placed in the heater block for exactly 20 minutes, and then immediately placed in cold water. The isomerised mixture was diluted with methanol and the extinction measured in a Unicam spectrophotometer at wave-lengths of 233, 268, 315, 346 and 375 mu.

Specific extinction coefficients were calculated, and the proportion of each polyethenoid acid in the mixture was deduced from Holman's (1957) formulae which are as follows:-

% Linoleic $1.087k_{233} - 0.615k_{268} - 0.1354k_{315} - 0.1072k_{346} - 0.412k_{375}$

% Linolenic $1.266k_{268} - 0.8028k_{315} + 0.3172k_{346} - 1.778k_{375}$

% Arachidonic $1.456k_{315} - 1.344k_{346} - 0.4128k_{375}$

% Eicosapentaenoic $1.599k_{346} - 1.628k_{375}$

% Docosahexaenoic $4.186k_{375} - 0.1778k_{346}$

'k' isthe specific extinction coefficient for the sample of mixed acids at the specified wave-length. It should be noted that the specific extinction coefficients quoted by this author are calculated for a concentration of g. per litre and not in g. per 100 ml. as in current British practice.

RESULTS

RESULTS

Evaporation of the petroleum ether solution from the initial extraction procedures yielded in each case an amber coloured oil with a pungent odour. The weights of mixed lipids recovered are tabulated below. (Table 1)

Table 1

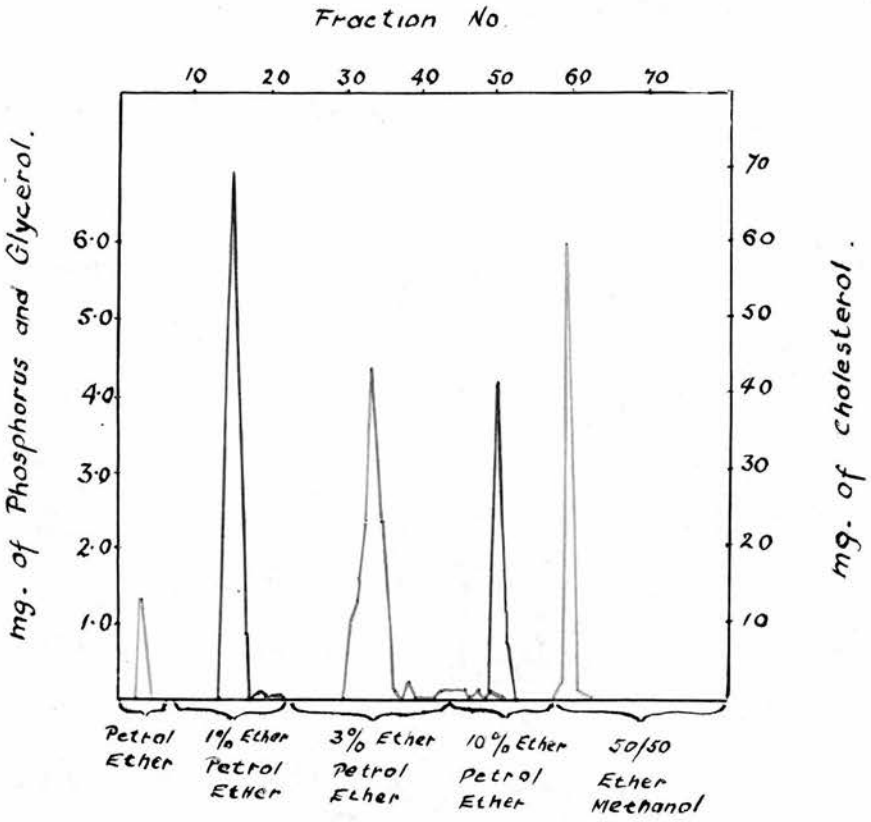
	Vol. of serum or plasma	Weight of crude lipid extract
Experiment No. 1	80 ml.	0.466 g.
No. 11	100 ml.	0.721 g.
No. 111	450 ml.	1.847 g.
No. 1V	25 ml.	0.127 g.

After standing for twenty-four hours or more in the refrigerator partial solidification took place and wheel shaped crystals appeared.

When the crude lipid was added to the silicic acid column in petroleum ether, a bright orange yellow band about 5 mm. deep was formed. A faint yellow band travelled down the column almost immediately and the first 100 ml. of eluate was coloured faintly yellow. A faint green band then separated and disappeared when the eluate was changed to 1% ether/petroleum ether. In most experiments, when petroleum ether containing 1% ether was applied a faint white opaque band could be seen travelling slowly down the column and its disappearance apparently coincided with the

Figure 6.

(EXPERIMENT II.)



Showing the elution pattern of mixed serum lipids on a column of silicic acid.

appearance of cholesterol ester in the effluent. In Experiment 11 a faint pink band separated very slowly from the top yellow band and faded; the remaining yellow band moved off on addition of 10% ether/petroleum ether and reached the lower end of the column as cholesterol appeared in the effluent.

Silicic Acid Chromatography of Lipids, Experiment 11

In the course of Experiment 11, 73 fractions of 50 ml. volume were collected. Fractions 1-6 were eluted with petroleum ether (B.P. 40°- 60°). Fraction 3 contained 6 mg. of a lipid substance which contained 2.2% of phosphorus and from which a trace of ammonia was liberated on incubation with urease; the remaining three fractions contained traces of a clear faintly yellow oil. Fractions 7-22 were eluted with 1% ether in petroleum ether and fractions 13-20 contained Liebermann Burchard positive material. Fractions 22-43 were eluted with 3% ether in petroleum ether, and fractions 30-36 contained a glycerol containing lipid. Fractions 44-56 were eluted with 10% ether in petroleum ether and fractions 50-51 contained Leibermann Burchard positive material. From fraction 57 onwards, the eluant consisted of 50% ether in methanol. Phosphorus-containing material was eluted in fractions 58-61 and no further lipid was eluted after this. The data can be seen expressed as a graph in Figure 6.

The phosphorus-containing lipid eluted in petroleum

ether was not observed in Experiment 1, where acetone had been used to precipitate phospholipids during the extraction. It was, however, found in the subsequent three experiments.

In Experiment 111, the lipid recovered from fraction 3 was examined; its phosphorus content was found to be 3.9% and after hydrolysis 3.2% of glycerol was found in the hydrolysate.

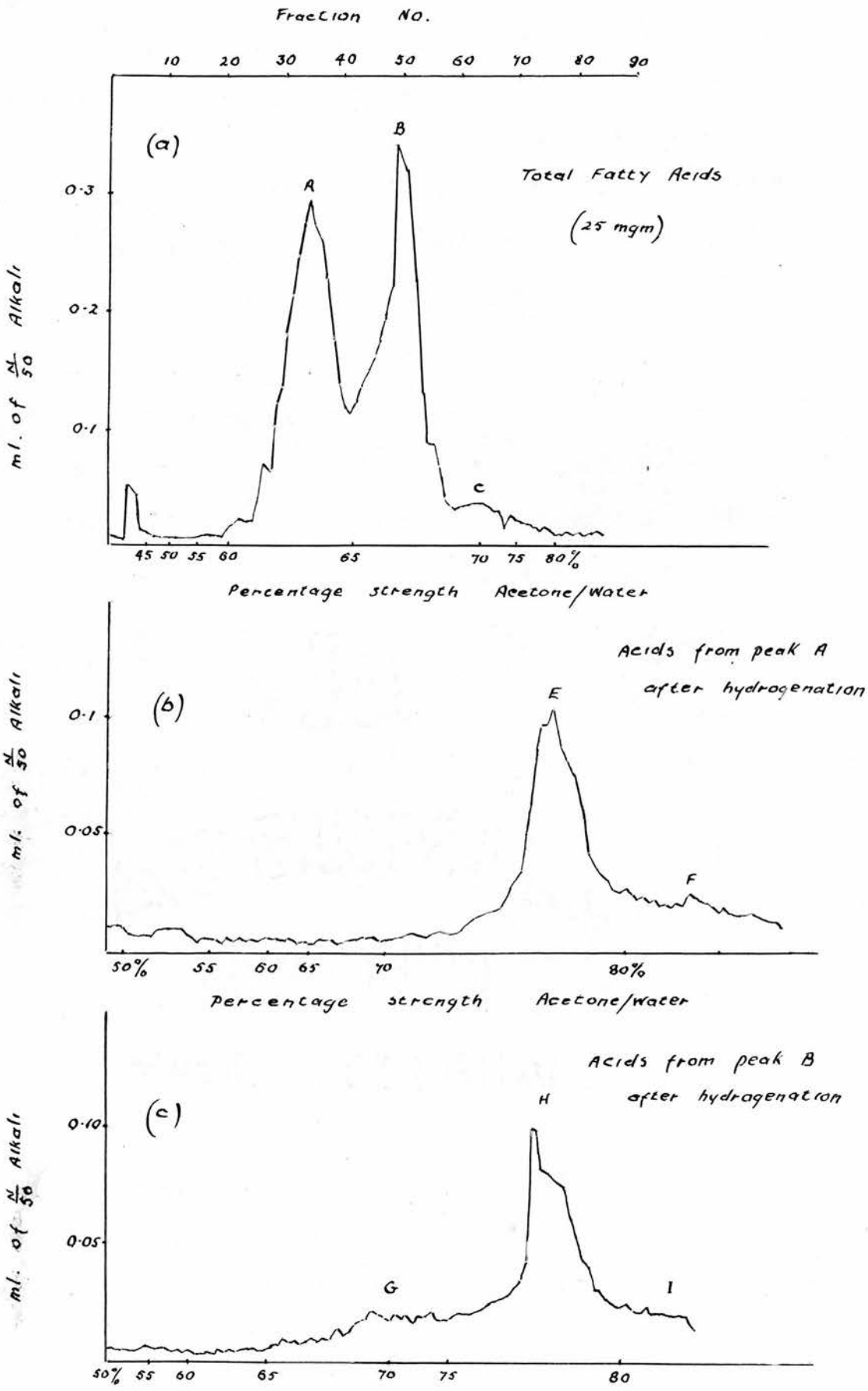
The cholesterol esters obtained from the silicic acid column were weighed and then hydrolysed and the total amount of fatty acid recovered from the hydrolysis was estimated by titration with 0.02 N. tetramethyl ammonium hydroxide with 0.1% bromothymol blue as indicator. The weight of acids recovered was calculated as oleic acid. A comparison of these recoveries with theoretical recoveries calculated fromthe digitonin analyses is shown in Table 11. The wide discrepancy between theoretical and observed weights of ester encountered in Experiments 1 and 11 was avoided in the later experiments by thoroughly washing the ester-containing fractions with water before distilling off the solvent. The poor yield of acids in Experiment 1 resulted from cautious extraction procedures performed at room temperature.

Table 11

		EXPERIMENT NO.			
		1.	11.	111.	1V.
Cholesterol levels as mg. per 100 ml. serum.	Total	136	147	105	156
Calculated as free cholesterol.	Free	54	56.2	35	62
From digitonin analysis.	Ester	82	90.8	70	94
Total cholesterol ester, calculated as mg. cholesterol oleate from digitonin analyses		111	153	529	39.6
Weight of ester (mg.) recovered from silicic columns		144	230	544	46
Theoretical weight of oleic acid (mg.) calculated from digitonin analyses		48	66	229.5	17.1
Acid recovered from hydrolysis (as oleic)					
	by titration	29.6	65.9	240	18
	by weight			236	

Experiment I Figure 7.

Reversed Phase Chromatography of fatty acids.



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The acids recovered from hydrolysis of the cholesterol esters in Experiment 1 were separated by reverse phase chromatography and the respective amount of each acid estimated by titration; the results, expressed as a graph, are shown in Figure 7(a). It can be seen that there are two large clearly defined peaks A. and B. eluted by 60% and 65% acetone respectively and a third smaller less well defined peak C.

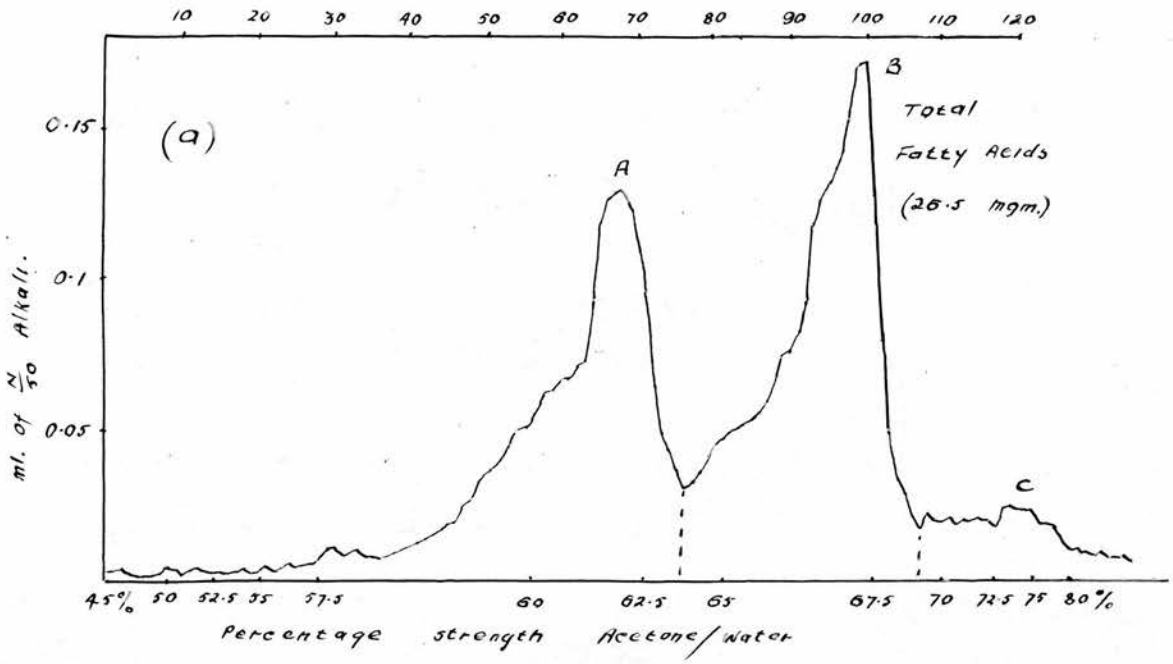
The fractions comprising peak A. were combined, and evaporated to small volume under reduced pressure in a stream of nitrogen; the concentrated liquor was made acid to congo red with 10% hydrochloric acid and the liberated acids were repeatedly extracted with petroleum ether. The pooled petroleum ether extracts were washed with water, dried over sodium sulphate and the solvent evaporated. The recovered acids were transferred in the minimum volume of ethyl acetate to the hydrogenation apparatus and hydrogenated for four days. Iodine numbers were estimated on a small aliquot of the hydrogenated acids and they were then again examined chromatographically. A well defined peak (E) eluted with 75% acetone, corresponding to stearic acid was noted, and also a small ill-defined peak (F) eluted with 80% corresponding to arachidic acid. These are shown in Figure 7 (b).

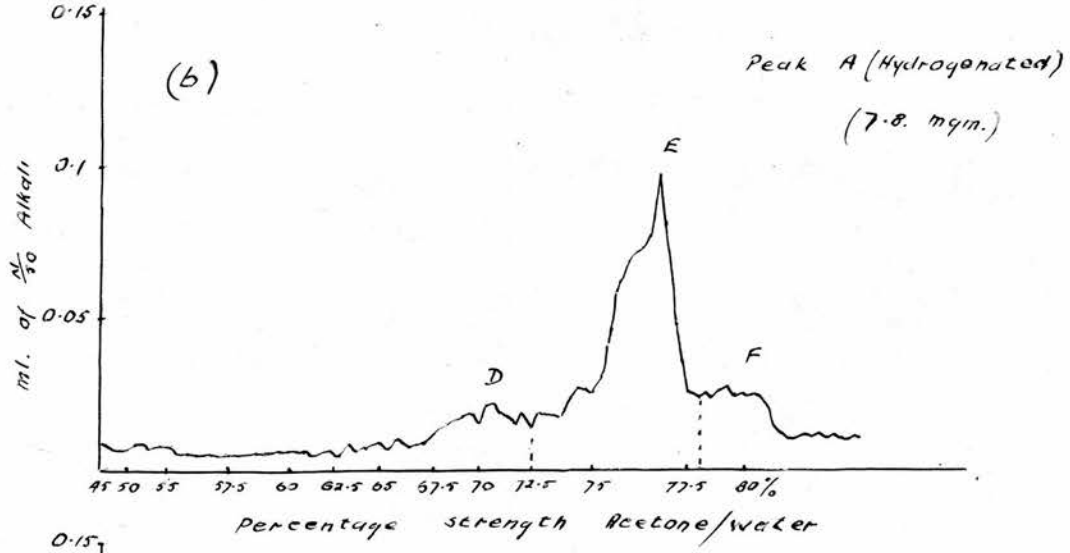
The acids from peak B. were similarly treated and the results of the titrations can be seen in Figure 7 (c). There is again a well defined stearic acid peak H. and ill-

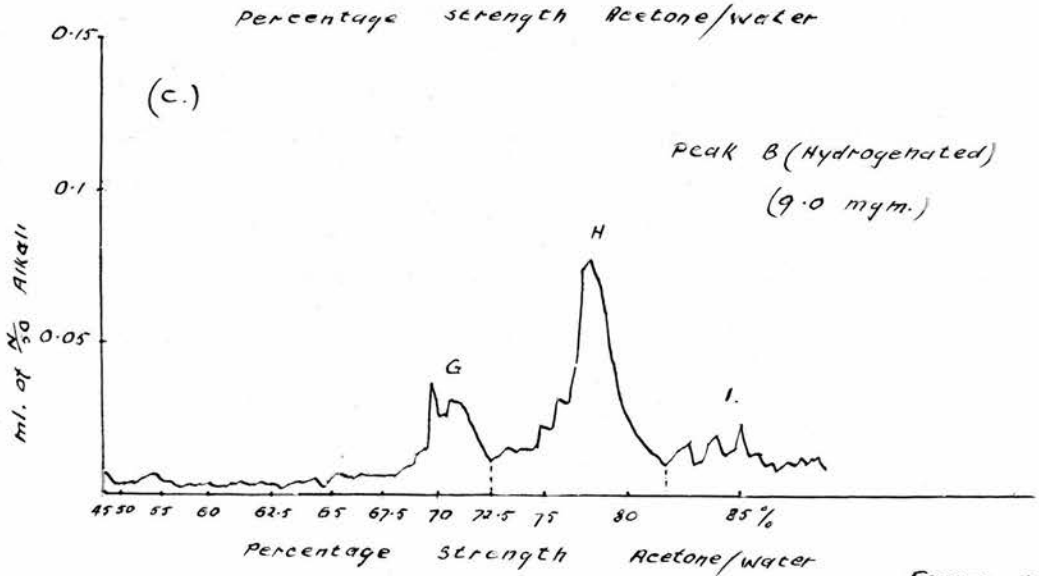
FIGURE 8

Experiment II

Reversed phase chromatography of fatty acids.







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defined peaks eluted in 65% acetone, corresponding to palmitic acid (G) and in 80% corresponding to arachidic acid (1).

Because of the poor separation obtained with the saturated acids, the columns were subjected to further study, and it was found that much firmer packing than indicated in the original papers resulted in better separation.

The acids obtained from hydrolysis in Experiment 11 were therefore examined on more firmly packed columns whilst the columns used for Experiments 111 and IV were rammed hard.

The results of chromatography of the total acids from the cholesterol esters in Experiments 11, 111 and 1V are shown in Figures 8(a), 9(a) and 10 (a). As in the first experiment, there are two large peaks A. and B. eluted in 60 and 65% acetone respectively and a smaller less well defined peak C. eluted in 70-75%.

As before, the acids comprising peak A. were extracted, then hydrogenated and the products chromatographed. Three peaks were observed, most clearly separated by the firmly packed columns used in Experiments 111 and 1V. As can be seen in Figures 8(b), 9 (b) and 10 (b) there is in each case a small peak (D) in the palmitic position (65% acetone) a larger peak (E) in the stearic position (75% acetone) and a smaller peak (F) in the position corresponding to arachidic acid (80% acetone).

FIGURE 9.

Experiment III

Reversed phase chromatography of fatty acids.

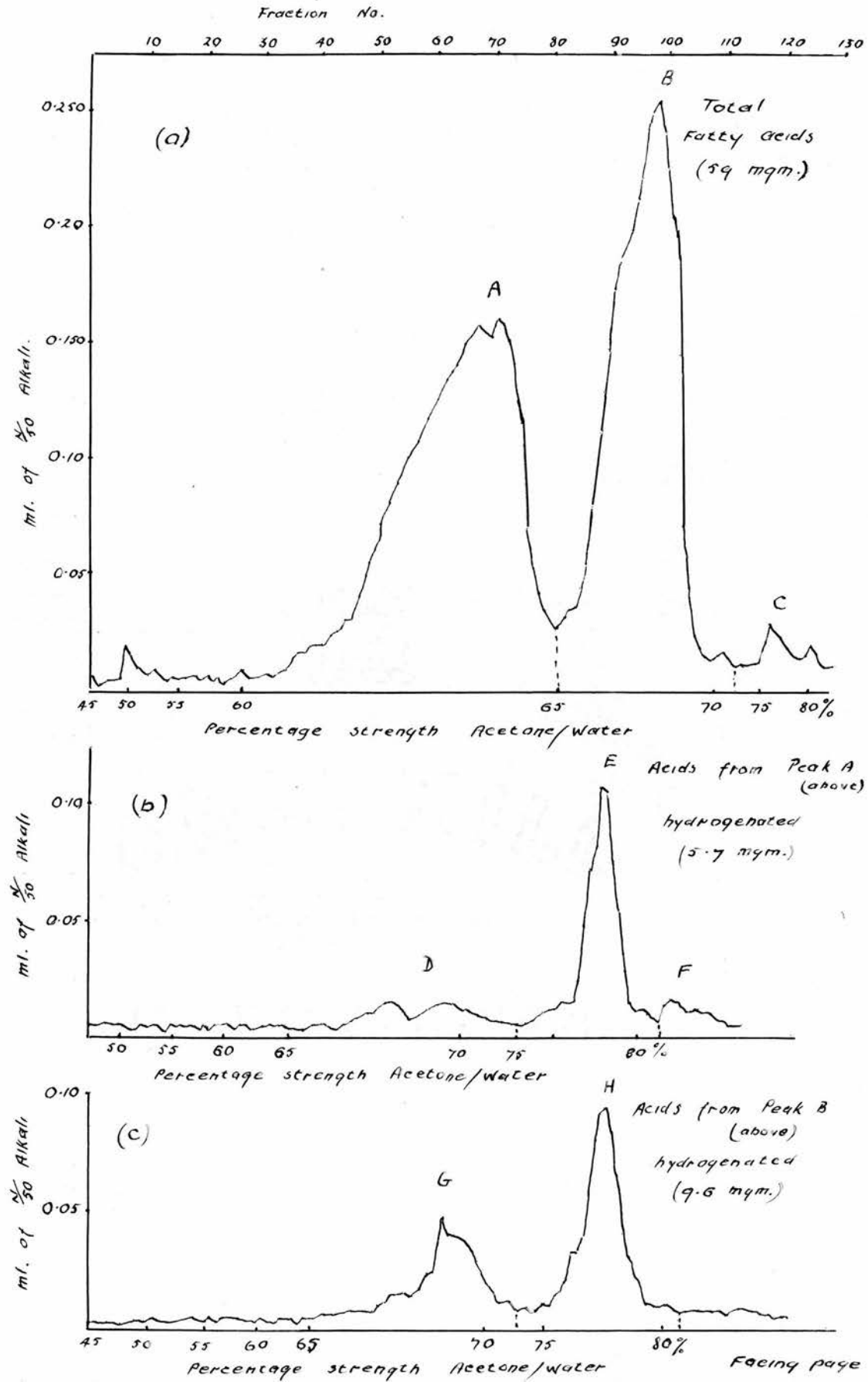

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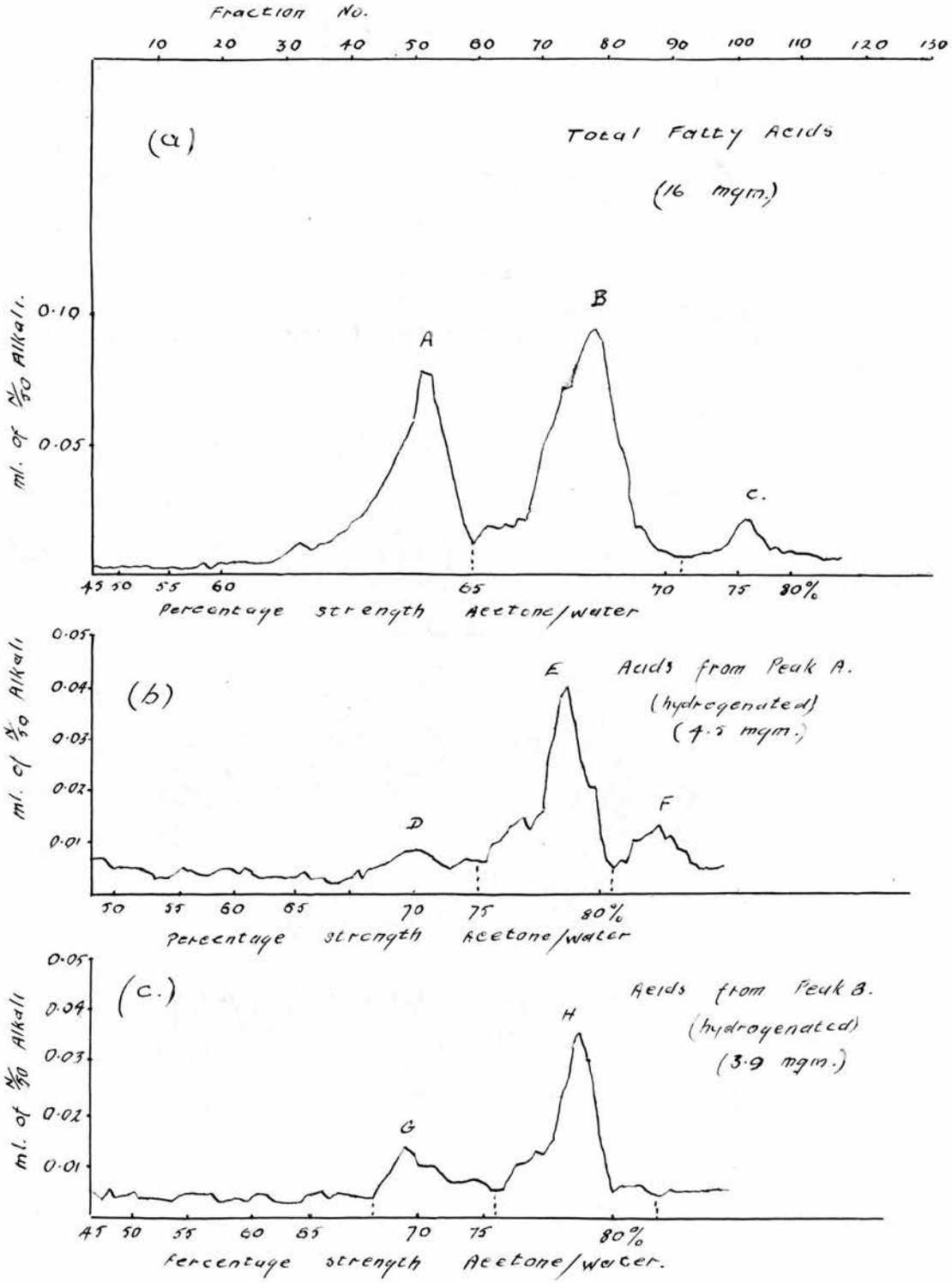
FIGURE 10.

Experiment IV

Reversed phase Chromatography of Fatty Acids.



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The acids recovered from peak B. were hydrogenated and chromatographed and the results are shown in Figures 8(c), 9(c) and 10(c). There are two clearly defined peaks, (G) and (H) in the palmitic and stearic positions respectively, and in addition, in Experiment 11, a third irregular peak (I) can be seen in the 80% acetone corresponding to arachidic acid.

The relative proportions of the acids isolated in these last nine chromatographic separations are shown in Table 111.

Table 111

Total Acids from Hydrolysis of Cholesterol Esters

		Peak A.	Peak B.	Peak C.	Recovery
Experiment	11.	38.4%	56.3%	5.3%	95%
	111.	50%	47%	3%	80%
	IV.	43%	53%	4%	89%

Acids recovered from hydrogenation of Peak A.

		Peak D.	Peak E.	Peak F.	
Experiment	11.	15.5%	66%	18.5%	93%
	111.	15%	78%	7%	95%
	IV.	9.5%	73.5%	17%	59%

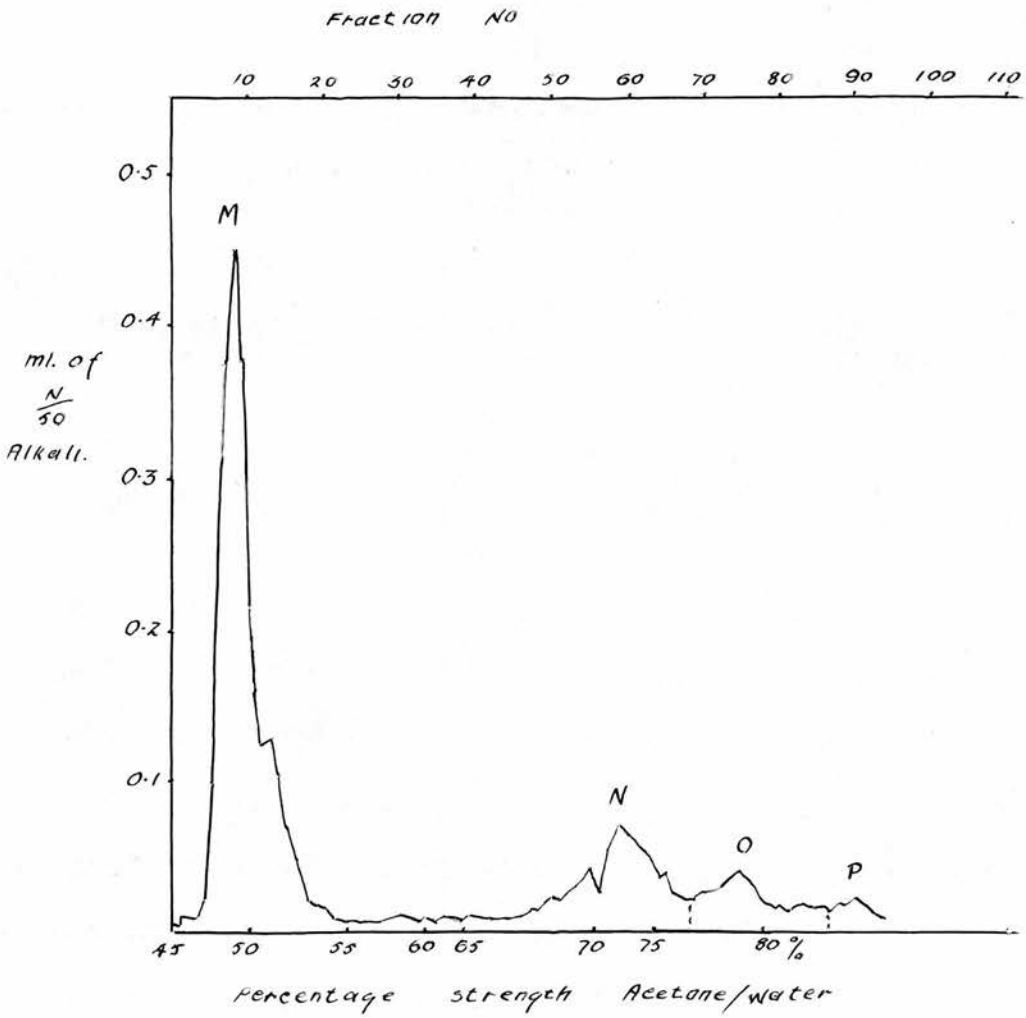
Acids recovered from hydrogenation of Peak B.

		Peak G.	Peak H.	Peak I.	
Experiment	11.	23.5%	60%	16.5%	84%
	111.	37%	63%	0%	86%
	IV.	32%	68%	0%	58%

FIGURE II.

Experiment II

Reversed phase Chromatography of Total Acids after Oxidation.



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Portions of the total acids recovered from hydrolysis in Experiments 11 and 111 were oxidised and after recovery were examined chromatographically. The results illustrated in Figures 11 and 12 show four peaks; a large peak M. comprising the oxidation products, and three small peaks, N., O. and P. derived from the unchanged, saturated fraction of the total acids and lying in positions corresponding to palmitic, stearic and arachidic acids respectively. The relative proportion of these acids is shown in Table 1V.

Table 1V

Relative proportions of acids separated by reverse phase chromatography after oxidation

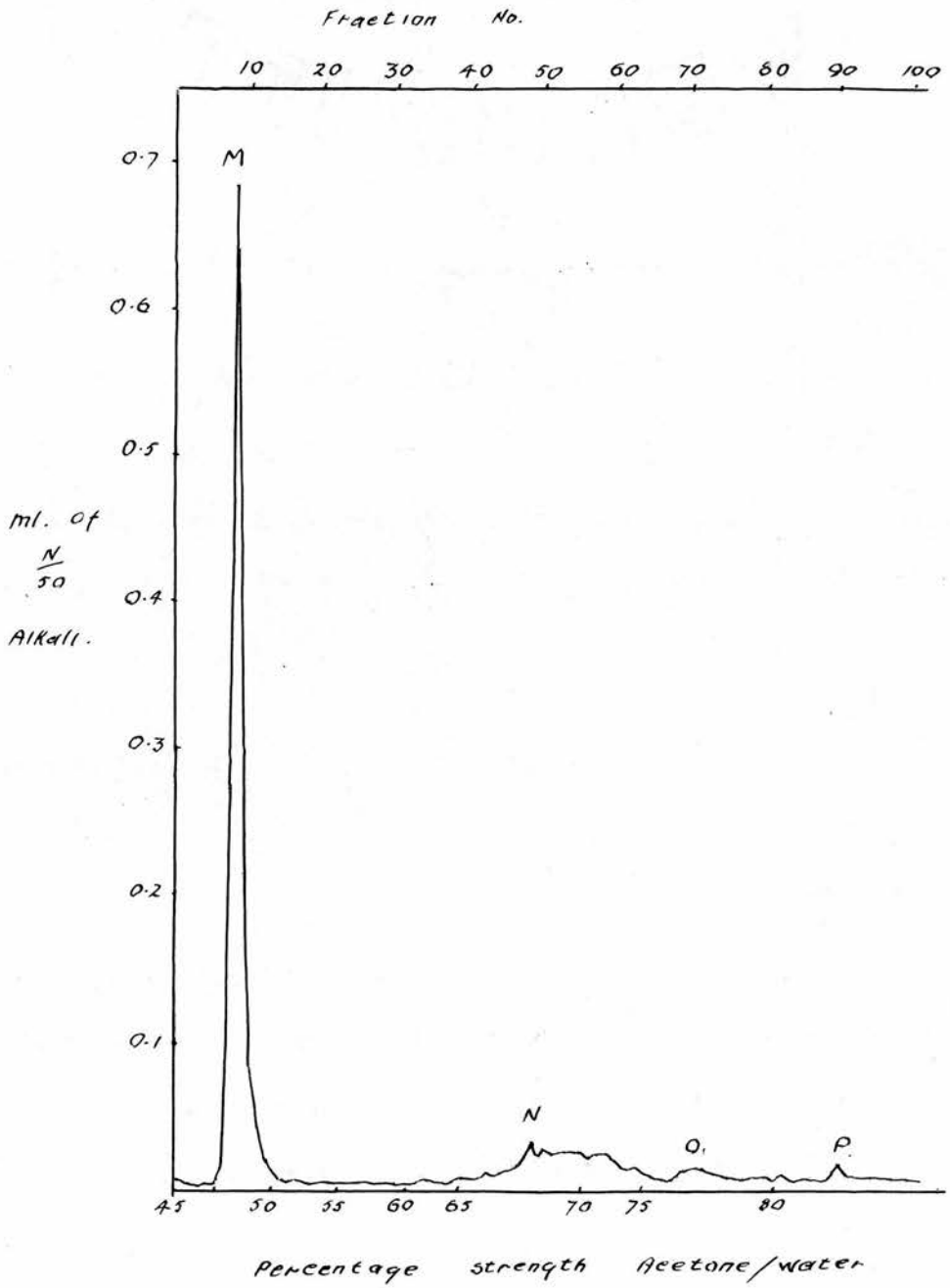
Peak M. (Oxidation products)	Peak N. (Palmitic)	Peak O. (Stearic)	Peak P. (Arachidic)	Total saturated acids
72.3%	18.4%	7.7%	1.6%	27.7%
72.8%	23.5%	2.7%	1%	27.2%

From the above data it can be seen that peak A. consists of acids containing 16, 18, and 20 carbon atoms and that the peak B. acids contain 16 and 18 carbon atoms (with a small amount of acid, containing 20 carbon atoms in Experiment 11). From the two oxidation experiments the percentage of saturated C16 acid recovered (peak N.) closely approximates the sum of the C16 acid present in peaks A. and B. These

FIGURE 12

Experiment III

Reversed Phase Chromatography of Total Acids after Oxidation. (24 mgm.)



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figures are derived as follows:-

Experiment 11

Peak A. - 38.4% of total

Peak B. - 56.3% of total

Peak D. - 15.5% of peak A. acids = 5.9% of total

Peak G. - 23.5% of peak B. acids = 13.2% of total

Sum of C16 acids ..... 19.1% of total

Peak N. - C16 saturated acids) .. 18.4% of total
in oxidation experiment)

Experiment 111

Peak A. - 50% of total acids

Peak B. - 47% of total acids

Peak D. - 15% of peak A. acids = 7.5% of total

Peak G. - 37% of peak B. acids = 17.4% of total

Sum of C16 acids .... 24.9% of total

Peak N. - C16 saturated acids).. 23.5% of total
in oxidation experiment)

From this, it is concluded that the C16 acidspresent in peaks A. and B. is palmitic acid, (though the possible presence of small amounts of hexadecenoic acid cannot be excluded).

The peaks C. lie in the position corresponding to stearic acid and their percentages of the total acids correspond fairly well withthe percentages of saturated C18 acids obtained in oxidation experiments.

The actual figures are:-

Experiment 11

Peak C. .. ..5.3% of total

Saturated C18 acid from oxidation experiment . ..7.7% of total

Experiment 111.

Peak C. .. .. 3.0% of total

Saturated C18 acid from oxidation experiment .. 2.7% of total

This suggests that peak C. is stearic acid.

The C18 acids found in peak A are eluted in 60% acetone. This behaviour is characteristic of linoleic acid. Similarly the C18 acids in peak B. are eluted in 65% acetone which is characteristic of oleic acid. The C20 acid found in peak A. must be even more unsaturated than the C18 acid and this must therefore be trienoic or tetraenoic. It is presumed to be arachidonic. In addition, the two oxidation experiments each show a tiny peak P. lying in the C20 position which indicates a trace of arachidic acid. This is probably concealed in peak C. in the chromatography of total acids. The irregular peak I (Figure 8(c)) noted in Experiment 11 represents a C20 acid occurring in peak B. and indicates an acid of relatively higher saturation than the C20 acid found in peak A.

From these data, relative proportions of the acids identified were calculated. In the case of Experiment IV, there was insufficient material for an oxidation analysis so the calculations were based on the assumption that all the

C16 acids were palmitic and that peak C. was composed of stearic acid.

The results areshown in Table V.

Table V

Relative proportions of cholesterol ester acids deduced from reversed phase chromatography (molar percentages).

	Experiment 11	Experiment 111	Experiment 1V
Oleic acid	33.7	29.6	36
Linoleic acid	25.3	39	31.6
Arachidonic acid	7.1	3.5	7.3
Other C20 unsat'd acids	9.3	-	-
Palmitic acid	18.4	23.5	17
Stearic acid	7.8	3	4
Arachidic acid	1.6	1	-
TOTAL	103.2	99.6	95.9

In Experiments 111 and 1V, iodine numbers were determined, both on the total acids recovered from hydrolysis and on the acids recovered from the combined fractions comprising the two main peaks A. and B.

From the titration data and the figures in Table V, theoretical numbers were calculated and compared with those obtained in Experiments 111 and 1V.

The results obtained indicate that the figures given in Table V of relative proportions of different acids are

approximately correct.

Table V1

Comparison of observed iodine numbers with values calculated from Table V.

	Experiment 111.			Experiment 1V		
	Total	Peak A	Peak B	Total	Peak A	Peak B
Theoretical value	108	161	56	111	183	61
Observed value	111	151	52.5	123	167	61

To confirm the nature of the polyethenoid fraction of the fatty acids, alkaline isomerisation was carried out by the method of Holman, on a portion of the acids recovered from hydrolysis in Experiment 111, and also on cholesterol esters isolated from a further sample of blood bank plasma. The conjugated isomers were examined in a "Unicam" ultra-violet spectrophotometer and the data obtained is shown in Table V11 in comparison with relevant figures obtained by chromatography.

Table V11

Polyethenoid component: proportion of total acids in Experiment 111

	By alkaline isomerisation	By chromatography
Linoleic acid	32.6%	39%
Linolenic acid	1.7%	
Arachidonic acid	3.9%	3.5%
Eicosapentaenoic acid	1.3%	
Docosahexaenoic acid	1.7%	
TOTAL	41.2%	42.5%

N.B. In the chromatographic experiments, a small amount of linolenic acid, if present, would be estimated as linoleic acid. The total C18 polyethenoid component by isomerisation is 34.3% and by chromatography 39%.

During the reversed phase chromatography of total acids in Experiments 1 and 111, small amounts of acid were eluted in 40-50% acetone. In Experiment 1 the ether extract of fatty acids liberated by the acidification of soaps was not washed with water; subsequently, it was found that neglect of the final washing invariably gave rise to such a peak and the smallpeak is, therefore, probably formed of hydrochloric acid. The column loading in Experiment 111 was very high (59 mg.) and the acids were loaded on to the column in acetone solution. This practice also, was found to give rise to occasional early peaks. A similar small

peak was observed by Lough and Garton (1957) in one of their experiments on cholesterol esters. In all other experiments no early peaks were found. With the exception of these two small peaks which are probably artefacts, no evidence of any acid of chain length of less than sixteen carbon atoms was found.

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DISCUSSION

1. Technical considerations
2. Physiological considerations
3. Proposals for future investigations

1. Technical Considerations

The extraction procedures gave recoveries of acid which agreed with those calculated from the digitonin estimation, within the limits of experimental error, except for Experiment 1 where temperatures were kept low throughout. This is despite the fact that the volumes of solvent used were much less than those found necessary by Boyd (1936) to give complete yields in single extractions. The multiple extraction procedure used here, appears to operate in two stages; the first treatment with a polar solvent, denatures protein and removes most of the water but apparently little lipid. The second extraction removes most of the lipid, and when the two extracts are pooled, a heavy precipitate is seen which is presumably the lipid in the second extract thrown out of solution by the water present in the first.

Since the difficulty in extracting cholesterol from serum appears to be due to it being bound to protein, it is likely that all that is required for efficient extraction is preliminary treatment with a small volume of a very polar solvent such as methanol, followed by two or three extractions with a good cholesterol solvent such as chloroform. There is little need for a complex extraction technique such as was used in Experiment 111.

Much of the trouble encountered in the adsorption chromatography of mixed fats on alumina and silica columns seems, in retrospect, to have been caused by the enormous

variability of adsorptive materials. The first attempts to separate cholesterol esters on alumina (Chromatographic) (British Drug Houses) gave results different from those obtained by Kerr and Bauld using alumina (Peter Spence) and by Clément et al (1954) (alumina unspecified). Greatly different results were obtained with Woelm alumina and of this variety the properties of the neutralised sample were found to be very different from those of the alkaline. Similar variations were found with silicic acid. Silicic acid (British Drug Houses) gave no separation of lipids, but silicic acid (Lights) behaved well in this respect. Garton and Duncan (1957) encountered similar difficulties in their work on bovine plasma lipids, and only obtained successful separations when they used silicic acid of American origin and prepared it in the manner of Borgström (1952).

The silicic acid columns used in the experiments described here, behaved differently from those of Fillerup and Mead in that they failed to adsorb a portion of phospholipids and Lovern (1956) has noted that lecithin is not adsorbed by silicic acid of British origin. A portion of the phospholipid eluted in pure petroleum ether in Experiment 111 yielded glycerol but only after prolonged alkaline hydrolysis in 25% potassium hydroxide in 50% ethanol at 100°C., and the yield of glycerol was only 25% of theoretical for lecithin. It was apparently a glycerophosphatide, the low yield of glycerol probably resulting from the difficulty in separating glycerol from the phosphoric acid radicle. This explains why no glycerol was recovered from

the small aliquots of phospholipid examined during the silicic acid chromatography in Experiment 11. Use of British silicic acid does appear to offer a method of separating phospholipids into two fractions since the non-adsorbed fraction must differ from that requiring methanol ether 1:1 for elution.

The whole question of adsorption chromatography of lipids requires closer examination. Silicic acid is obviously a very valuable material and it seems likely that its activity could be varied by graduated wetting as was done by Brockman and Schodder (1941) with alumina.

The identification of fatty acids in small quantities only became practicable when the reversed phase technique was developed by Howard and Martin (1950). The method undoubtedly gives clear separation of fatty acids and the position in which they are eluted gives strong evidence as to their identity.

Recovery of acids from the column seems to vary with the operator. Crombie et al (1955) and Howard and Martin obtained recoveries of 95-97% whilst Silk and Hahn (1954) using a very refined technique, obtained recoveries of only 60-80%. In this work recoveries have ranged from 60% to 97%, being generally poorest when very small amounts of lipids were examined. The error seems to lie in the titration since no accumulated fatty acids were found on their columns by Silk and Hahn, and columns can be used

repeatedly without any variation in behaviour. The end point of the titration becomes indistinct with larger amounts of fatty acids, obviously due to buffering by the soaps that are formed and since titrations are performed in varying amounts of acetone, water and liquid paraffin it is understandable that the results may not be accurate.

Reversed phase chromatography is too tedious and requires too much material to be of major value in physiological experiments, but the invention of vapour phase chromatography has provided a much sharper tool. With this method complete analyses of complex mixtures of fatty acids can be performed on as little as 2 mg. of material in under an hour.

The hydrolysis of cholesterol esters both for the digitonin estimation of total cholesterol and for recovery of fatty acids was carried out by prolonged heating with an excess of 20% sodium ethoxide. Preliminary work on the hydrolysis of cholesterol esters in this laboratory indicates that cholesterol esters of saturated acids are much more resistant to hydrolysis than those formed of unsaturated acids. The milder techniques in general use, such as that of Schoenheimer and Sperry (1934) which employs potassium hydroxide at a final concentration of about 0.5 N. and a temperature of 40°C. for 30 minutes, may fail to hydrolyse all or part of the saturated fraction.

The failure of alkaline permanganate oxidation to

reduce the iodine value to zero is at variance with the findings of Crombie et al (1955). There are several possible reasons for this.

It is possible that C20 polyethenoid acids are incompletely oxidised by this method (in Crombie's work, no acid of chain length greater than C18 was examined). Alternatively, the oxidation products of polyethenoid acids may react with free halogens. A clue to the explanation, however, appears to be provided by the observation that Hubl's method of iodination gave an iodine value approximately twice that obtained with Rosenmund Kuhnhenn reagent. Hubl's technique employs mercuric chloride to catalyse the reaction and it has recently been observed (Planck et al 1953) that addition of mercuric acetate to Rosenmund Kuhnhenn reagent ensures full bromination of elaeostearic acid which is otherwise difficult to halogenate.

Gelber and Boeseken (1929) demonstrated that Wij's reagent (iodine monochloride) adds halogen quickly to one of the double bonds in 9, 11 linoleic acid and to two of the three conjugated double bonds in elaeostearic acid. In both cases however, halogenation is only complete after two to three days. Neither in Crombie's experiments nor in this series was it known how much conjugation had been induced in the unsaturated acids by the alkaline hydrolysis of esters or the alkaline permanganate treatment.

In addition, it does not appear to be known whether the presence of hydroxyl groups interferes with halogenation. Halogenation of an ethylenic double bone probably occurs by virtue of the halogen molecule becoming polarised in the polar solvent in which the reaction is carried out, the polar halogen causing induced electron displacement in the chain close to the double bond. An adjacent hydroxyl group might be expected to oppose such an electron movement. It was thought possible that incomplete oxidation of some of the polyethenoid acids might occur and that for the above reason, halogenation might not go to completion.

In order to gain some insight into this problem a sample of castor oil B.P. was purified by repeated extraction with petroleum ether, followed by passage down an alumina column, and its iodine value estimated by Yasuda's (1931) method, firstly as originally described and then with the addition of 1 ml. of 2.5% mercuric acetate in glacial acetic acid. The iodine values obtained were 42 and 99.5 respectively. There is thus little doubt that the Rosenmund Kuhnhenn reagent is unable to fully brominate the triglyceride of 12 hydroxyoleic acid except in the presence of mercuric acetate.

It seems, therefore, that iodine numbers do not provide a reliable index of the degree of unsaturation following oxidation with alkaline permanganate.

Since the iodine numbers are not reduced to zero,

the method is left to some extent uncontrolled, but it is highly unlikely that any unsaturated acid would escape at least partial oxidation during the prolonged treatment employed.

The acids identified in these experiments differ significantly from those found by Lough and Garton (1957) in bovine plasma, the principal difference being the small amount of linolenic acid in the cholesterol esters of the human. This is not an unexpected difference since linolenic acid is an important constituent of grass lipids (Hilditch, 1956a) but is virtually absent from the human diet.

In the reversed phase chromatography of fatty acids described by these authors the first peak was eluted in 55-58% acetone, whilst their second peak in 60% acetone corresponds to the first peak (A) shown in the present work. Their first peak contained the linolenic acid and in addition small amounts of myristic, palmitic and arachidonic acids. It is interesting to note that small subsidiary peaks can be seen in Figures 1a, 2a and 4a of the present work, eluted before the main bulk of peak A. These are presumably composed of arachidonic and probably a small amount of linolenic acids. This peak is not visible in peak A in Figure 3 (a) but only 3.5% of arachidonic acid was found in this experiment, and moreover, the column was heavily loaded.

The iodine numbers noted by Lough and Garton, i.e. 189.5 are, as would be expected from the presence of linolenic acid, correspondingly higher than those found in the present experiments (111-125). These last are also slightly lower than those found by Clément et al, (130) and much lower than those found by Bloor et al (1937-38) (157.8). The method of separating ester fatty acids used by Bloor and his co-workers (that of Kelsey and Longnecker, 1941) is not entirely specific. Dietary differences between the U.S.A. and Britain may also partly account for the difference. Recently Lewis (1958) has examined the fatty acids attached to the cholesterol circulating in the blood of South African Bantus and whites, by alkaline isomerisation after separation of the cholesterol esters on silicic acid columns. He found mean iodine numbers of 179 and 160 for healthy Bantus and whites respectively and the corresponding polyethenoid acids (molar percentages) were 82% and 71.7%.

This worker's results show higher percentages of polyethenoid acids and higher iodine numbers than were found in the present work, which again may be due to dietary differences in the two populations. In this connection it is interesting to see that the specimens from Bantus showed appreciably higher proportions of polyethenoid acids than those obtained from whites. The problem of complete hydrolysis is again also present. In the South African work outlined above, hydrolysis was performed with

potassium tert-butoxide at 35°C. and the saturated esters may not be entirely hydrolysed by this technique.

The finding in Experiment 11 of peaks in the C20 position after hydrogenation of peak B. (Figure 11.C) is of interest. No evidence of any corresponding acid was found in the other experiments. It might conceivably be an artefact, but since the serum used was obtained from patients in an acute general hospital, the possibility that it is an abnormal constituent cannot be ignored. The study of the fatty acid pattern in disease may well yield much information.

2. Physiological Considerations

It is first necessary to consider the present knowledge of the cycle of cholesterol. Over the last few years it has been shown that cholesterol is synthesised from short chain fatty acids in the liver (Bloch, 1942, Popják, 1949, Cornforth, 1953). The liver apparently furnishes a supply of cholesterol to the plasma at a fairly constant rate (Bloch, 1950). A portion (apparently a fixed fraction of that synthesised) is secreted in the bile (Peters and Van Slyke, 1946a).

The biliary cholesterol (which is the free alcohol) mixed with dietary cholesterol (which contains little or no ester) is partly absorbed, the absorption being entirely by the intestinal lymphatics (Chaikoff et al, 1952). The process is a slow one (Biggs et al, 1951) and the quantity of sterol absorbed is modified by the amount of fat in the

diet, restriction of fat causing a reduction in cholesterol assimilation.

The lymph in the intestinal lymphatics and thoracic duct contains a mixture of free and esterified cholesterol in the same ratio, one to the other, as they are in the blood stream (Mueller, 1915, 1916).

The cholesterol circulating in the blood in health remains remarkably constant in level and since the rate of synthesis does not appear to be modified by dietary change, the level must be controlled either by excretion or destruction. Excretion of cholesterol in health is almost entirely by the intestine (Adlersberg, 1951, Sperry, 1932, Sperry and Angevine, 1932). Variations in the plasma levels are only followed by increased intestinal excretion when the changes are very large.

Destruction of cholesterol in the body is known to occur, much being converted into cholic acid and excreted in the bile (Bloch et al, 1943, Byers et al, 1952).

Since the liver constantly manufactures and destroys cholesterol the variable effect of parenchymatous liver disease may be understood; mild disease may be presumed to interfere with the destructive process so that the plasma cholesterol rises, whilst more severe disease interferes with synthesis as well, so that the plasma level falls.

From the cycle outlined above, it is clear that at

least some esterification of cholesterol takes place in the intestine, in fact it appears to be a requirement for efficient absorption of the sterol.

The saturated fatty acids in the diet as judged from the data provided by Hilditch (1956 b) exist in a wide range of chain lengths, the lower chain lengths being largely confined to dairy products in which significant amounts of n. decanoic, lauric and myristic acids occur. On the other hand, unsaturated acids of chain length shorter than C16 are very minor constituents of the diet, and hexadecenoic acid itself is almost confined to liver, milk fat and ground-nut oil. If esterification of cholesterol took place entirely in the intestine, one would expect to find saturated acids with chain lengths lessthan C16 amongst the esters.

In these experiments no acid having lessthan sixteen carbon atoms has been observed. Even if the small peaks observed in Experiments 1 and 11 were not artefacts, no peaks corresponding to lauric or myristic acids were found. The unsaturated acids identified, namely, oleic, linoleic and arachidonic (and possibly small amounts of linolenic and hexadecenoic) constitute almost the entirety of the dietary unsaturated acids. This evidence suggests that cholesterol combines only with unsaturated acids in the intestine.

The oleic acid in food-stuffs greatly exceeds in

amount the linoleic so that if the esterification were unselective, one would expect the oleic ester to exceed that formed from linoleic acid but in these experiments approximately equal proportions of the two acids were observed. It follows that the esterification of linoleic acid is preferential, and one may suppose that the two double bonds facilitate the enzymic esterification of cholesterol.

The esters of cholesterol with unsaturated fatty acids have much lower melting points than either cholesterol itself or the esters formed with saturated acids, and this alone may be the factor determining the absorption of cholesterol from the gut. It is well known that the harder, more saturated triglycerides are poorly emulsified and poorly absorbed and this may hold good for cholesterol also.

The question arises as to the point of origin of the saturated esters. It has been shown that an esterase exists in blood plasma, active at 37°C. which esterifies cholesterol at the expense of the phospholipids (Sperry, 1935, 1936, Tayeau & Nivet, 1956), during which process the iodine number of the cholesterol esters falls. This suggests that the phospholipid supplies a saturated acid. Phospholipids which have lost one fatty acid possess haemolytic powers, this being the effect of the enzyme lecithinase found in cobra venom (Lovern, 1955 b). This toxic effect is reduced or neutralised by the presence of a sufficiency of cholesterol (Bloor, 1943 b) which may well

act by providing a receptor for the second acid. These facts offer some evidence that cholesterol may receive a portion of its saturated acids in the bloodstream.

Cholesterol esterases have also been demonstrated in the liver and it is possible that saturated esters are formed thare and released into the bloodstream though there is nothing to indicate what purpose this would serve.

Such evidence as there is supports the view that the formation of saturated esters occurs in the plasma. It is noteworthy that cholesterol in the plasma is largely bound to α and β globulins and it is possible that this sterol protein complex, as far as the free cholesterol is concerned, represents an enzyme complex permanently available to take up instantaneously any fatty acid that may be set free in the bloodstream. If this supposition is correct, free cholesterol may be a specific detoxicating agent for free acids or soaps reaching the bloodstream.

It has been repeatedly observed (Bronte-Stewart et al, 1955, Gordon et al, 1957, Malmros et al, 1957) that modifications of the dietary fats affects the serum cholesterol. The feeding of highly unsaturated fats over a prolonged period lowers the blood cholesterol level and also lowers the iodine numbers of the esters (Kinsell et al, 1958). It appears that when there is an adequate supply of polyethenoid acids, they are preferentially esterfied, an occurrence that would be expected if esterification was a function of the number of double bonds in

the fatty acid; having been absorbed, the polyethenoid esters are more readily metabolised.

The feeding of fats containing a large proportion of saturated acids has the reverse effect; with large amounts of saturated acid reaching the blood stream there may be an increased requirement for the receptor activity of cholesterol, and it is fair to assume that the saturated esters are less well metabolised. This last is supported by work now in progress in this laboratory, which indicates that the bond strength of esters with saturated acids is much greater than is the case with unsaturated acids. This would add a further possible reason for the rise in blood cholesterol on a diet of saturated fat.

The evidence suggests that cholesterol serves as a vehicle for transporting the highly unsaturated acids of the diet from the intestine to the bloodstream, and onwards to some unidentified destination, which is probably the liver. A subsidiary function, involving the free cholesterol, appears to be that of a detoxicating agent active towards any free fatty acid, soap or undesirable acid fraction arising from the catabolism of complex lipids.

3. Proposals for Future Investigations

Two distinct lines of approach suggest themselves, the one clinical, the other physiological.

With regard to the clinical studies, the present work suggests that the pattern of the fatty acids esterified with cholesterol does not vary widely in health except as it may be modified by prolonged changes in the diet. The finding of an abnormal C20 acid in Experiment 11, together with Lewis's (1958) observation that the proportion of polyethenoid acids esterified with cholesterol is much lower in subjects with coronary disease than in the healthy indicates that there may be considerable alteration of the pattern in the diseased state.

It is obvious that there is an urgent need to demonstrate the pattern of these fatty acids in disease, but an accurate picture of the normal pattern in a wide range of individuals is an essential prerequisite for such a study. Once this is established, work can be directed towards the consideration of the diseased pattern. With this end in view, a vapour phase chromatography apparatus is under construction, both to expedite the work and also to scale it down to the point where it is practicable to perform the analysis on volumes of blood that can reasonably be taken from sick people.

The alternative, physiological line of approach is suggested by the indications given in this work that the unsaturated acids are esterified with cholesterol in the intestine and saturated acids are esterified elsewhere.

If this supposition is correct, the feeding of a

single large dose of a pure unsaturated acid, either as its ethyl ester or as its triglyceride would be expected to give an early change in the pattern of the cholesterol ester fatty acids. Conversely, substitution of a saturated acid for the unsaturated acid in the above experiment would be expected to cause a much later and smaller change.

If changes of pattern were found with experiments of this type, it wouldthen be desirable to compare the effects of acids of different degrees of unsaturation, and of acids not normally present in the diet.

Such experiments would entail the repeated withdrawal of blood samples and wouldof course require vapour phase chromatography. It would probably be necessary to examine simultaneously the fatty acid pattern of the other acid-bearing lipids to gain some idea of the destination of such acids as failed to be esterified with cholesterol.

It is hoped that preliminary work on these lines will be undertaken concurrently with the clinical studies outlined earlier.

CONCLUSIONS.

1.

In two samples of human serum, and one sample of human plasma the fatty acids of the cholesterol esters were of C16 chain length or longer. More than 70% of these acids were unsaturated.

2.

The different acids identified, together with their molar percentages were as follows:-

Oleic acid	(29.6 - 36%)
Linoleic acid	(25.3 - 39%)
Arachidonic acid	(3.5 - 7.3%)
Palmitic acid	(17 - 23.5%)
Stearic acid	(3 - 7.8%)
Arachidic acid	(1 - 1.6%)

There is also some evidence of the presence of the following acids:-

Linolenic acid	(1.7%)
Eicosapentaenoic acid	(1.3%)
Docosahexaenoic acid	(1.7%

A small amount of hexadecenoic acid may be present.

3.

During esterification of cholesterol in the intestine, the more unsaturated acids are used in preference to the less unsaturated; the double bond may play some direct part in the process.

4.

The saturated acids are probably not esterified with cholesterol in the intestine. This process may occur in the bloodstream.

SUMMARY

1.

The fatty acid componentsof the human serum cholesterol esters have not been previously identified. This is due to the difficulty of separating cholesterol from the other lipids present in serum and of identifying small quantities of fatty acids.

2.

This ignorance has hindered both the understanding of the function of cholesterol and the development of satisfactory methods for the estimation of its level in the serum.

3.

In this work, three samples of human serum and one of plasma were examined; two of the specimens were from healthy donors, the other two were each made up of combined small samples from twenty-five patients in the acute wards of a general hospital.

4.

The lipids were extracted from these specimens by conventional methods, and the cholesterol esters isolated by adsorption chromatography on columns of silicic acid.

5.

The fatty acids obtained by hydrolysis of these cholesterol esters were examined by reversed phase chroma-

tography on columns of medicinal paraffin, aqueous acetone being used as the eluant. Portions of the acids were examined before and after hydrogenation; thus information regarding their chain lengths and degree of unsaturation was obtained. In a further portion, the unsaturated acids were destroyed by oxidation with alkaline potassium permanganate and the remaining saturated acids identified by reversed phase chromatography.

6.

Attention is drawn to the variability of materials used for adsorption chromatography. The importance of firm packing of the stationary phase used in reversed phase chromatography is stressed.

7.

The major constituents were found to be oleic acid (29.6 - 36%), linoleic acid (25.3 - 39%), arachidonic acid (3.5 - 7.3%), palmitic acid (17 - 23.5%), stearic acid (3 - 7.8%) and arachidic acid (1 - 1.6%). No acid of chain length lessthan 16 C was observed. Minor constituents identified by alkaline isomerisation included linolenic acid 1.7%, eicosapentaenoic acid 1.3% and docosahexaenoic acid 1.7%.

8.

The relative proportions of acids found are significant; the high proportion of linoleic acid suggests preferential esterification of the more unsaturated acids

by the intestinal enzymes. It is possible that these may require the presence of a double bond in order to operate.

9.

Since the dietary fat contains saturated acids of chain length less than 16 C, the absence of such acids in the cholesterol esters is regarded as evidence that the saturated acids are not esterified in the intestine. It is suggested that such esterification may take place in the bloodstream.

10.

For future work, it is proposed to make, with the aid of vapour phase chromatography, a wide study of the normal serum cholesterol fatty acid pattern in all ages and both sexes, followed by a study of the pattern in disease. In addition, it is proposed to study the effect of doses of a single fatty acid on all the fatty acid bearing blood lipids.

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